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# THE CONSERVATIVE PATHOLOGIST AND THE MORPHOLOGY OF TUMORS PRODUCED BY THREE CANCER VIRUSES IN MICE[a]

THELMA B. DUNN[b]

## *Morphology of Viral Tumors in Mice*

Nearly every week I read in the newspapers that the prevention and cure of cancer are just around the corner. And the reason given for this optimistic prediction is that we now know that cancer is caused by a virus, and all we need do is find an effective vaccine, as we have for poliomyelitis, or discover another miracle drug like penicillin. Then cancer will vanish from the human race. I hate to question such glowing prophecies, because a wet blanket is not held in high esteem, and the man who puts on the brakes usually gets a jolt. But let me remind you that a wet blanket is handy to have around when a fire threatens to get beyond control, and every well-equipped vehicle needs reliable brakes.

Andrewes (1) recently stated: "Most pathologists . . . don't like the idea that cancer has an organism as its cause. . . . And now the filtrable mouse leukemia and the polyoma have shaken even the most orthodox." Andrewes is perfectly right. The orthodox pathologist is perhaps the last holdout against the growing enthusiasism for viruses as *the* cause of cancer. This reluctance has a well-rounded tradition and was clearly expressed by Ewing (7) in 1928. He wrote: "The parasitic theory is the oldest hypothesis of the origin of cancer. It appealed to the ancients, was tacitly accepted throughout the middle ages, was definitely argued by modern observers, and reached the height of its popularity about 1895, but during the last 15 years it has rapidly lost ground and today few competent observers consider it as a possible explanation of the unknown element in blastomatosis."

[a] Received for publication November 20, 1960. Presented before the Southwestern Section of the American Association for Cancer Research.

[b] Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland. National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

I now introduce myself to you as a conservative or orthodox pathologist. I am a devout disciple of Nicholson (15), and Nicholson's philosophy, like many other inspired gospels, cannot be distilled into a few words. Perhaps this quotation comes closest: "Tumours, in their structure, their functions, and the manner of their growth, do not differ essentially from other tissues, and obey the laws that govern their behavior. In no essential character do tumours differ from the other tissues of the body." To put this in a nutshell—there is nothing in a tumor contrary to nature.

Let me conclude this introduction with the statement that I am not a virologist; far from it. I am like one of my colleagues who ruefully admitted that he perhaps knew less about viruses than many virologists do about cancer.

The pathologist is limited in his scope. He relies to a great extent upon that antiquated instrument, the light microscope, which in some circles is even considered obsolete. He must admit that many things do go on in a cell beyond his range of vision. The alterations which become morphologically manifest in a cell are extreme. Profound chemical and hormonal changes can be present in a cell that appears unchanged to the pathologist's limited vision.

The pathologist, however, remains aware of the disease. There is so much concentration now on the study of cancer viruses, that the disease manifestations they produce are being ignored. Let me quote again, this time from Willis (22): "The tubercle bacillus does not cause tuberculosis in a test tube, or in some species of animals, or even in some human beings. Tuberculosis involves two reagents, the bacillus and the susceptible tissue into which it is introduced."

I will now describe to you three disease processes, or cancerous conditions, which I, a conservative pathologist, must admit followed the introduction of a cell free substance presumably containing a virus into the tissues of susceptible mice. I have had the privilege of working closely with Howard Andervont on milk-agent tumors, with Lloyd Law and Clyde Dawe on a variety of tumors produced by the parotid tumor agent, and more recently with John Moloney on leukemia induced by his agent. In the first part of my talk I shall confine myself strictly to what I have personally observed, or information I have obtained first-hand from those whom I consider to be reliable wit-

nesses. My hope in doing this is to impress you not so much with what is known, as with what is as yet unknown, and needs to be found out. After that, I want to speculate, but I shall make it entirely clear when I am switching from fact to fancy. Even if viruses are proved to be a primary cause of cancer, if one reflects upon the complexities of the three systems I plan to discuss, he will admit that we have only just begun the fight for the prevention and cure of neoplastic disease.

First, let us consider mammary tumors of mice (5). These are perhaps the most completely studied neoplasms in existence. Newborn mice of a low mammary cancer strain, if foster nursed on mice of a high mammary cancer strain have a greatly increased incidence of mammary tumors, and these tumors appear at an early age. To show the changing times, this foster nursing effect was first called a milk influence, next a milk factor, then a mammary tumor agent or inciter, but now it is boldly referred to as the mammary tumor virus. This virus is highly specific. It produces only mammary tumors in mice, and so far as anyone can discover, this change in mammary tumor incidence is the only effect it has. Strain C3H mice with the virus appear to be identical to strain C3H mice without the virus. I have made some fumbling attempts, with the assistance—perhaps I should say indulgence—of Dr. Andervont to find a difference in mice with and without the virus, first by comparing peripheral blood studies and autopsy findings of infant mice into which the virus was injected, with blood and tissues from non-injected litter mates in the early post-natal period. The treated mice showed no pathologic effects that I could detect. Proceeding to an older age group, around 6 months, I compared the endocrine organs of mice with and without the agent, and detected no difference. However, these efforts were crude and I never published the results. Counts of various types of pituitary cells might have shown a numerical variation. Dr. John Bittner (3) has stated that the agent may alter the hormonal pattern of the host. It is an excellent possibility, and I hope he can produce the proof.

The first morphologic difference to be found in mice with the mammary tumor agent is the appearance of hyperplastic nodules in the mammary glands of female mice. The same type of nodule will also appear in smaller numbers and at a later age in female mice without the agent. In mice with the agent, many

of the nodules progress to cancer; in mice without the agent only a few nodules appear and undergo a neoplastic change. When tumors from mice with the agent are examined microscopically, most of them exhibit a glandular pattern. When the few tumors developing in mice without the agent are examined, tumors of a similar glandular pattern will still be found, but there will also be an appreciable number of tumors of more unusual types. The age difference is probably important, but no age matched controls are available, since the mammary tumors cause an early death in the agent bearing mice.

I will now show lantern slides to illustrate these points:*

1. Adenocarcinoma, Type A, composed of tissue resembling mammary gland acini. This type is especially frequent in strain C3H mice with the agent.

2. Adenocarcinoma, Type B. This type also shows a glandular structure, and is common in mice with the agent.

3. Adenocanthoma. This type shows both glandular and squamous elements. It has been found frequently in mice without the agent treated with methylcholanthrene.

4. Carcinosarcoma. Here we have a mingling of epithelial carcinoma cells, and fusiform sarcoma cells. This type also has been seen most often in mice without the agent after painting with methylcholanthrene.

5. Adenocarcinoma, Type C. This type is comparatively rare. It has been found most often in older mice without the agent. It consists of small cysts lined by cuboidal epithelium surrounded by a layer of fusiform cells, which are probably myoepithelial. This tumor has a complex structure.

6. Organoid Tumor. I call your attention particularly to this tumor. The central area is composed of squamous epidermal tissue, with radiating ducts of epidermal cells, and marginal glandular tissue. It is reproducing all the structure of a mammary gland, with the epidermal tissue of the nipple appearing centrally. When transplanted, the organoid tumor maintains the same complex structure as the primary tumor, although only a small mass of cells is introduced into the recipient mice. These organoid tumors are one reason pathologists are reluctant to accept viruses as a universal cause of cancer growth. I cannot

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* Photographs of these lesions and the chart appear in reference 11.

credit viruses with enough intelligence to reproduce all the structures of an organ.

7. Chart, showing incidence of tumor types in mice with and without the agent. Note that Types A and B are common when the agent is present.

Although Heston, Andervont, and Bittner have expressed the same idea, Mühlbock (14) has stated in very clear terms our present position on the action of the milk agent in inducing the glandular mammary tumors in mice. He regards the virus as an accelerator or intensifier in the histogenesis of a type of tumor that could develop without it.

Chronologically a discussion of the parotid tumor agent, or the polyoma virus, should come next, but this is so complex, I want to leave it until last. Dr. John Moloney and I have recently completed a study of his leukemia virus (6). Moloney (13) recovered a virus from a transplant of Sarcoma 37 and this virus when injected into mice produced leukemia after a period of months. The same virus could then be recovered from the induced leukemia tissue. The virus infects both newborn and adult mice and rats. In our joint experiment, Dr. Moloney injected the virus into the mice and I performed the autopsies and studied the tissues histologically. The mice were all BALB/c males six weeks of age when injected. An extract from leukemic spleen was given intraperitoneally to one group and an extract from the spleens of normal mice was given to controls. The mice were autopsied in groups of 5, first at daily intervals, then at weekly or more prolonged intervals. The first difference between experimental and control mice was detected at 8 weeks. Something must have occurred earlier, but I was relying upon the crude methods of a pathologist using a light microscope and I could find no difference in the two groups. A more discriminating method should have revealed a difference, for the virus was certainly at work during thees 8 weeks, but silently like a phantom. This failure to find an early morphologic change was disappointing to me, for I can readily comprehend how a virus might produce an acute disease from which an animal could recover, while a preneoplastic change persisted and led later to overt cancer. At 8 weeks, however, a series of changes began to develop and could be detected. I can best illustrate these with lantern slides: *

* These slides are all made from photographs in reference 6.

1. Table showing that 44 mice survived the 8 weeks period. Of these, 9 showed widespread lymphocytic neoplasms, 9 showed lymphosarcoma usually limited to the thymus, and 24 showed preneoplastic changes. Two mice appeared normal.

2. Granulocytes within megakaryocytes in the bone marrow. This was the earliest difference detected between the control and experimental mice. It was not, of course, pathognomonic of the virus infection, since it appeared occasionally in the control mice, and it has been found in other experimental mice. However, it was frequent enough in the virus treated mice to make it possible to separate the bone marrow slides from controls with a high degree of accuracy.

3. Splenic hyperplasia. Beginning about 8 weeks, there was a gradual enlargement of the spleen, and it often reached 3 to 4 times the normal size. Histologically, this hyperplasia affected all elements, and an increase in megakaryocytes was especially prominent. When leukemic infiltration occurred, the spleen became 10 or more times normal size, and the normal architecture was obscured by the neoplastic cells.

4. Thymic atrophy. This could be determined only by close comparison with the controls, since it was not more extreme than may appear in old mice, or mice subjected to a variety of unfavorable conditions. Thymic lymphocytes which are normally concentrated in the cortex of the thymus, were often so reduced that a medullary type of tissue reached to the capsule.

5. "Potter lesion" in lymph nodes. A lesion which we refer to as the "Potter lesion" was described by Potter and associates (17) as a preneoplastic lesion in strain C58. A similar lesion was found in three of our virus treated mice. Nodular areas were found in lymph nodes, and within these nodules there were nests of large reticulum cells surrounded by normal appearing lymphocytes.

6. Early thymic leukemia. The slide shows one lobe of the thymus enlarged and replaced by neoplastic cells, while another lobe is atrophied. This thymic alteration is remarkably similar to the appearance of the thymus in the X-ray induced leukemia described by Kaplan (10), and the early thymic changes in the spontaneous leukemia of strain AKR/O described by Arnesen (2).

7. Granulomatous lesion in thymus. This is a remarkable alteration in the thymus, and I do not recall ever seeing a lesion

just like it before. Granulomatous nodules can be seen composed of large foam cells in which "cholesterol" clefts appear. This thymic alteration was found only once in a virus treated mouse. The nearby tracheo-bronchial nodes were leukemic and leukemic tissue appeared elsewhere. This was the only instance in which leukemia did not begin in the thymus.

8. Full blown leukemia showing greatly enlarged thymus. This appears to be a typical lymphocytic neoplasm of the mouse, beginning in the thymus, and rapidly involving nearby structures.

9. Microscopic detail. The microscopic appearance of this leukemia was likewise typical. Large phagocytic cells filled with nuclear debris are prominent.

One question we wished to decide was whether the leukemias induced by the Moloney virus would all be identical histologically and by cell type and whether they would behave in the same fashion on transplantation. Many observers have noted that each transplantable leukemia line in a mouse usually has some quality of growth or organ infiltration by which it can be distinguished from other lines. So tissue from 8 enlarged spleens of the Moloney virus induced leukemia was transferred to 5 mice each, and the morphology and behavior of the resulting neoplasms was studied. Morphologically all were lymphocytic neoplasms, but they differed in their rate of growth, the degree of dispersion of the malignant cells, and the organ involved. One line, for example, appeared 10 days after inoculation and always involved the adrenal medulla; another line appeared after 20 days and involved the epididymis. Although these were all lymphocytic neoplasms, they were not identical.

An unexpected result was obtained when enlarged spleens from four other virus infected mice were used for transfer. On histologic examinations carried out later, these spleens were found to be hyperplastic, and not neoplastic, for they contained no leukemic cells. The donor mice inoculated with bits of tissue from these enlarged spleens did not develop a lymphocytic neoplasm either generalized or localized at the site of transfer, even though they were kept under observation for from three to four months. Instead, they showed the same complicated series of tissue changes that folowed upon the inoculation of the Moloney virus alone. In two of the spleen inoculated mice, the process

reached the ultimate stage and a primary lymphosarcoma appeared in the thymus. In other words, and to put it briefly, we were transferring a virus which had multiplied in the spleen before any leukemic change had appeared. The virus effects, therefore, can be found although the neoplastic change is absent.

Subsequent work with the Moloney virus has shown that the onset of leukemia is greatly delayed in thymectomized or splenectomized mice, and that under these circumstances, unusual forms of leukemic disease appear, such as granulocytic leukemia or reticulum cell neoplasms. We have also observed a curious granulomatous reaction in the thymus of mice injected with virus many weeks previously, and then irradiated or given some chemotherapeutic drug such as myleran or amethopterin, just when leukemia was beginning to develop. This lesion is like the one I showed in the photograph. The Moloney virus may, like the milk agent, prove to be an accelerator and an intensifier of tumor formation, and the thymus may be an accessory factor in this process, just as estrogenic stimulation is for the mammary tumors induced by the milk agent. But much more needs to be learned about the Moloney virus before any opinion can be hazarded. At the present time we are handicapped because our information on pre-leukemic conditions in the spontaneous disease is limited.

I now come to the most complex system of all, and that is the parotid tumor agent or the polyoma virus. The discovery and manifestations of this virus appear to me completely factitious, a laboratory tour-de-force, that would never have been disclosed in mice that were left alone. And although we hear that the polyoma virus is so widespread, as witnessed by the studies of Dr. Huebner and his associates (19), it might disappear tomorrow, and neither the pathologists studying spontaneous mouse tumors, nor, I suspect, the mice themselves, would be any the wiser.

This virus was first disclosed when an extract of AKR leukemic tissue was injected into newborn strain C3H mice by Dr. Ludwik Gross (9). I collaborated with Dr. Lloyd Law in an investigation of this agent in 1954 (11). In this early investigation the virus material was obtained directly from a variety of transplanted and primary tumors in AKR or C58 high leukemia strain mice, and it was injected into newborn C3H mice from

Dr. Law's colony. The yield of parotid tumors was less than ten percent and they occurred in mice averaging nearly a year in age. A remarkable feature was that the tumors were not randomly distributed but clustered in litters. In one experiment, when hybrid C3H Heston mice by the C3H Figge line were used, in addition to parotid tumors, subcutaneous neoplasms were also found and one tumor of the adrenal medulla. The parotid tumors were pleomorphic, and unlike any I had ever seen before or heard described in mice. Tumors originating in the exorbital lachrymal gland were also frequent, while the submaxillary and sublingual salivary glands were rarely involved. Two of the subcutaneous tumors in the flank in male mice were also extraordinary since they closely resembled parotid gland tumors, and were histologically unlike the usual mammary tumors.

In a later collaboration with Dr. Clyde Dawe and Dr. Law (4) the material for injection was obtained by Dr. Dawe from the tissue culture growth of a variety of leukemic lines. Although a few tumors resulted when the supernatant fluid from leukemic cell cultures was injected, it was only after a tissue culture line called P388 was deliberately infected with the parotid tumor agent obtained from a parotid tumor, that a high incidence of parotid and other tumors appeared at an early age.

In this later investigation the litter effect also apeared, and it was found that the mothers that produced resistant litters gave a positive antibody reaction to the virus, and presumably this was transmitted to the young through the placenta and in the mothers' milk, and this made the young mice resistant. Dr. Mearl Stanton and associates (21) at the National Cancer Institute also observed that the offspring of mothers from an environment infected by the polyoma virus were more resistant to the oncogenic effect of the agent. This introduces a paradox that as gaily mocks at commonsense as any devised by Gilbert and Sullivan (16). The best way to insure that her offspring *will* have mammary tumors is to infect the mother mouse with the mammary tumor agent. The best way to insure that her offspring *will not* have polyoma type tumors, if the virus is injected in the newborn, is to infect the mother mouse with the polyoma virus.

In the second experiment, when the parotid tumor agent had been fortified by passage through tissue culture, numerous

tumors were observed not seen in the first experiment, and also a cytologic effect, especially evident in the cells of the kidney tubules and bronchial mucosa.

These lantern slides will illustrate some of the lesions:*

1. Chart showing different tumor incidence after treatment with two different tissue culture preparations. This chart was prepared by Dr. Clyde Dawe. It shows on the left the sites involved in neoplasms and other lesions in 20 consecutive mice that developed tumors of the parotid glands after inoculation with short-term tissue cultures of a leukemia line received from Ludwik Gross. On the right were the sites involved after inoculation with first passage of P388E tissue cultures with the parotid tumor agent. Note that many more tumors were found in the mice on the right. Much more needs to be learned about the effects of preparations from different sources in inducing tumors, and also the variations in the number and types of tumors induced in mice of different genetic background.

2. Myoepithelioma of the parotid gland. I show this slide because it represents a tumor of the parotid gland that develops spontaneously in untreated mice, especially in strains A and BALB/C. We have seen it frequently, and it must be distinguished from tumors induced by the parotid tumor agent. This is a cystic growth filled with a characteristic slimy fluid.

3. Parotid tumor from a virus treated mouse. This slide represents a characteristic parotid tumor induced by the agent. It is multicentric and pleomorphic, and entirely different from the spontaneously occurring parotid gland neoplasm just shown.

4. Mammary tumor. This represents a mammary tumor found in a male mouse treated at birth with the parotid tumor agent. It shows an intraductal growth, and is unlike the mammary tumors that I showed you earlier and that we customarily find in mice. Moreover, we have not previously found mammary tumors in untreated male mice, yet mammary tumors of an unusual type appeared frequently among the males in some groups of mice receiving the parotid tumor agent at birth.

5. Thymic tumor. In our experience, true thymomas, that is, tumors derived from the thymic epithelium, are extremely

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* Photographs from which the lantern slides were made can be found in references 11 and 4.

rare in mice. Yet tumors developing from epithelial tissue in the medulla as shown in this slide, were common in some groups of the agent treated mice.

6. Bone tumor. The bone tumors we have previously observed in mice appeared almost exclusively in strain C3H. They have a characteristic pattern, with many osteoclasts. After treatment with the parotid tumor agent, strain A mice develop osteomas and osteogenic sarcomas unlike those found in strain C3H. The one shown here shows an osteoid type of tissue without osteoclasts.

7. Hair follicle tumors. We frequently see epidermoid carcinomas after painting the skin of mice with methylcholanthrene. We also find basal cell carcinomas, and tumors of the sebaceous glands. However, multiple hair follicle tumors, such as I show in this slide, have not been encountered before. It looks as if this parotid tumor agent or polyoma virus has gone out of its way to produce skin tumors of a new type.

8. Adrenal medulla. We frequently find tumors of the adrenal cortex, especially in certain strains of mice after gonadectomy. We also find pheochromocytomas of a distinctive pattern within the medulla. But the tumors of the adrenal medulla found in the agent treated mice histologically resemble some adrenal cortical tumors, and are not the previously known pheochromocytomas. Our whole previous experience with neoplasms in the mouse is upset.

9. Renal tubular lesion. This lesion shows a swelling or ballooning of nuclei in enlarged cells of the kidney tubules. Although this startling alteration is found at this site, neoplasms of renal epithelium have not been found. A similar lesion has been found in bronchial mucosa, but it is not followed by lung tumors. It is astonishing, not only that new types of tumors appear after the parotid tumor agent injection at birth, but also that many characteristic neoplasms of the mouse, such as alveolar pulmonary tumors and hepatomas are not increased. The chemical carcinogens usually increase the incidence of tumors that would appear spontaneously. This parotid tumor agent has introduced an entirely new spectrum of tumors in the mouse.

Now let us make some comparisons among these three virus systems, and more or less summarize our present knowledge about them.

*First*—regarding the tissues from which the virus in these three systems can be recovered. The most important source is shown in italics for each of them:

The mammary tumor agent—
1. *Milk of infected mothers.*
2. Mammary tumors.
3. Other organs of infected mice.
4. Seminal fluid.
5. (Laboratory transfer doubtful.)

The Moloney leukemia virus—
1. Sarcoma 37.
2. *Virus induced leukemia.*
3. Infected but not yet leukemic spleen.
4. (Laboratory transfer doubtful.)

The parotid tumor agent—
1. Leukemic tissues from AKR and other strains.
2. *Tissue cultures especially if infected by the virus.*
3. Normal tissues, especially from AKR mice.
4. From the induced tumors, but with difficulty. (Laboratory transfer—usual.)

Second, let us consider the types of lesions produced in mice by these three viruses:

Mammary tumor agent—
1. Hyperplastic nodule.
2. Mammary adenocarcinomas.

Moloney leukemia virus—
1. A "derangement" in reticular cells and organs.
2. Lymphocytic neoplasms.
3. Other reticular neoplasms in operated mice.

Parotid tumor agent—
1. New forms of parotid gland and other tumors.
2. Nuclear alteration.
3. Runting (in some experiments).

Third, let us compare the methods by which the virus or tumor is transferred: Mammary tumor agent—Affects mice only. *Virus* is transferred by foster nursing, coitus, and injection. *Tumors* are readily transferred to members of same inbred strain.

Moloney leukemia virus—Mice and rats so far have been infected. *Virus* is transferred by injection to mice and rats, both

newborn or adult. *Tumors* are readily transplantable in the same inbred strain.

Parotid tumor agent—Affects mice and other rodents. *Virus* is transmitted by contact, and by injection to mice and other rodents. *Tumors* are produced only by injection, and are difficult to transplant.

Now that I have tried honestly to put the facts of these three systems before you, I ask your indulgence while I question and speculate.

I would like to ask first how the conservative virologist, if such there be, reconciles these observations with Koch's postulates. I know that Rivers (18) in a paper on "Viruses and Koch's Postulates" has said that these laws do not have to be fulfilled in order to prove that a virus is the cause of a disease. He adds, however, that the spirit of Koch's rules of proof still holds since a worker must demonstrate that a virus is not only associated with a disease but that it is actually *the* cause. If Rivers had said *a* cause, one could argue no further about these systems. But we have yet to find a situation in mammalian oncology where a virus can be recovered from a particular type of tumor, and from that type of tumor only, and injected into another animal and reproduce the same type of neoplasm, and that only. Rivers, toward the end of his paper, predicted that progress in virology would be limited only by the ingenuity of investigators, but he warned that to obtain the best results this ingenuity needed to be tempered by the priceless attributes of common sense, proper training, and sound reasoning.

In the dilemma in which the conservative pathologist is placed, I have come up with a scheme—I will not call it a subterfuge—by which I can admit the existence of cancer viruses and at the same time keep my allegiance to Nicholson. Most pathologists are willing to recognize that a virus could be another carcinogen—after all, cancer inducing agents and cancer provoking conditions are already innumerable. But the idea that any cell would allow a virus to come in, take over its domestic arrangements and so dominate the parent cell and all its progeny that it formed a new breed unmindful of normal regulation is more than the conservative pathologist can now accept. To him, it is against all reason, whether teleological or mechanistic. Here is my resolution of the conflict:

1. Tumor viruses do not directly invade and alter a living, progenitive cell.

2. Tumor viruses invade only dead or dying cells, or cells with no future. In other words, the tumor virus is a scavenger, not a predator.

3. Tumor viruses, in conjunction with the products of dead cells, create an extracellular environment in which the normal cell is altered and becomes neoplastic.

I have already discussed the first point. What evidence is there for the second? It is this: the tissues or conditions from which tumor viruses are recovered or in which they multiply always involve many dead or dying cells, or cells at the end of the road. In the three systems under discussion, these are:

1. Milk. This should be an ideal medium, for the lactating cell is doomed to quick extinction. It commits suicide in the performance of its function.

2. Neoplastic tissue. I am always nonplussed when a chemist asks for a tumor "free from necrosis." I have never seen one. Besides the large areas that can be located and removed, my colleague, Ross MacCardle has pointed out that "single cell necrosis" is very abundant, especially in Sarcoma 37. We know also from the work of Levaditi and Nicolau (12) and many later investigators that viruses may seek out cancer tissue as a preferred site in which to multiply and thereby cause damage to the tumor.

3. The newborn state. We are so impressed by the building up and construction that takes place in fetal life that we forget that it is also a time of breaking down and demolition. If a contractor built a house the way nature makes a baby, we would be sure his blueprints were cockeyed, or else he was being supervised by a woman who could not make up her mind.

4. Tissue culture. In the type of maintenance cultures that virologists carry, dead and dying cells are always lying about, and a cytopathogenic effect greatly increases the number. Also remember that cells in tissue culture are in a precarious, and unnatural state. Normal cells grown in tissue culture have always been shown to become neoplastic eventually, if they can be adequately tested, whether or not virus was added to the culture (20).

Now we come to the crucial question that justifies all the at-

tention given to cancer viruses in animals. This may be phrased in different ways, depending upon the speaker. The conservative will ask "Are any human cancers ever caused by a virus?" The more liberal "Are some human cancers regularly caused by a virus?" While the real radical wants to know "Aren't all cancers in man or animals caused by a virus?" I belong to the first group, and with all the straining and striving now going on, I anticipate that someone will recover a virus from the cancer tissue of a human being and that when such artificial procedures as a sojourn in tissue culture and inoculation into newborn animals have been performed this virus will produce some form of disease or even cancer. But what will be the value of that? My answer is "A great deal." Nothing has so enlivened cancer research as Gross's discovery of a virus in AKR leukemia tissue in 1951 (8). Let us never discourage a search for viruses in human cancer! As long as the search is made in good faith by reliable workers who are not shackled to a theory, they should have unstinted support in money and equipment. There cannot be too much cancer research, and every stimulus to it should be augmented. We do not always find what we are looking for, but what we do find may be something of greater value. Remember that Columbus was looking for India when he discovered America. If he had known what he was going to find, he might never have started out, and if Queen Isabella had been like some of the granting agencies, she would never have given him the money. Then where would we be today?

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# THE FUTURE OF MEDICAL EDUCATION IN TEXAS

## *The need for more physicians*

EDWIN G. TROUTMAN[a]

For some years there have been many popularly quoted opinions and publications regarding the need for more physicians. In only one of these reports, "Physicians for the South" by Dr. Winfred L. Godwin of the Southern Regional Education Board (8), has the status of physician supply and medical education in Texas received more than indirect attention. In Dr. Godwin's report Texas is shown to have a potential deficit of 5000 physicians by 1975.

Beginning with the famous and remarkable "Flexner Report" (7) of 1910 which delineated the then current status of medical education in the United States and Canada, there has been a succession of books, articles, and reports. Mr. Flexner's original publication is recommended to the interested reader who would like to read for himself the shocking and deplorable conditions of that era when medical education was often a business for personal profit, even to the formation of stock companies paying dividends. Following Flexner's survey there was drastic and immediate change. Some schools died as a result of the Carnegie Foundation-supported revelation of their true status. Others reformed so swiftly that Mr. Flexner had to revise his final draft to report only the new status which usually reflected a major change for the better.

In the subsequent two decades, medical schools of stable and sound foundation eventually began to turn out enough graduates so that by 1930, and for twenty years thereafter, the supply of physicians in the United States increased at a faster rate than the population (14). In 1949, the peak year of physician supply, there were 130 physicians per 100,000 population in the United States. This dropped to 129/100,000 by 1959.

The 1959 Texas physician/population ratio was 101/100,000 —lower than that of six other Southern states. Only one Southern

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state—Maryland—had a ratio above the national average of 129/100,000. Maryland's ratio of 138/100,000 was still lower than the 141/100,000 enjoyed by that part of the United States outside the South (Table 1).

The expected urbanization of Texas between now and 1975 will exceed that of the South as a whole (8). It is anticipated that by 1975 85% of Texans will live in cities and towns—an increase of 21% in the next 15 years! As a result, the already serious maldistribution of physicians in Texas rural counties may drop below the 1957 ratio of 66/100,000 (compared to the metropolitan county ratio of 107/100,000).

This plight of our rural counties will be partially offset by at least two factors. First, as urbanization of Texas proceeds, each rural county will find itself closer to an urban area. It has been estimated that in Mississippi, supposedly the most rural of the Southern states, any patient can reach modern medical facilities within a half-hour (9). Secondly, the trend towards group practice coupled with the construction of many new modern medical facilities will attract full and part-time specialists to the smaller urban areas where they will be more readily available to the non-urban patient population. In fact, rural patients may even be able to reach a group of specialists more rapidly now than they could have reached a rural general practitioner three decades ago.

Neither of these factors—urbanization nor the expected trend to group practice—will significantly offset the total availability of physicians in rural areas if our present physician/population ratio of 101/100,000 cannot be maintained.

### *Physician Supply in Texas*

The deficit of 5000 physicians for Texas estimated in the report from the Southern Regional Education Board is based on high estimates of supply, and represents the anticipated difference between available physicians and the number necessary to reach and maintain a ratio equal to the 1959 national average of 129/100,000. *It would take the output of five new four-year medical schools producing 100 graduates each year from 1966 to 1975 to erase this deficit!* And to graduate a physician in 1966 means he has to enter the first year class in 1962. These "high estimates of supply" made by the Southern Regional Education

TABLE 1

*Admissions to Medical Schools in Each Southern State Showing Numbers in Public-supported Schools and Private Schools With Out-of-state Admissions Compared, 1959*

| State (D.C. excepted) | Ratio of Physicians/ 100,000 in 1959 | Total Admissions in State | Admissions to Public-supported Medical Schools | | | Admissions to Private Medical Schools | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total | Nonresidents | % of Admissions Nonresidents | Total | Nonresidents | % of Admissions Nonresidents |
| Maryland | 138 | 178 | 100 | 23 | 23.0 | 78 | 69 | 87.5 |
| Florida | 122 | 130 | 49 | 3 | 6.2 | 81 | 5 | 6.2 |
| Delaware | 119 | 0 | * | ... | ... | † | ... | ... |
| Louisiana | 114 | 260 | 128 | 0 | 0 | 132 | 111 | 84.0 |
| Tennessee | 106 | 255 | 203 | 53 | 23.0 | 52(2) | 34(2) | 65.5 |
| Virginia | 102 | 160 | 160(2) | 58(2) | 36.2 | † | ... | ... |
| TEXAS | 101 | 336 | 252(2) | 7(2) | 2.8 | 84 | 37 | 44.0 |
| Oklahoma | 100 | 101 | 101 | 2 | 2.0 | † | ... | ... |
| Arkansas | 97 | 92 | 92 | 2 | 2.2 | † | ... | ... |
| Georgia | 95 | 179 | 102 | 0 | 0 | 77 | 39 | 50.6 |
| North Carolina | 95 | 200 | 69 | 6 | 8.7 | 131(2) | 76(2) | 58.0 |
| Kentucky | 88 | 99 | * | ... | ... | 99 | 17 | 17.2 |
| West Virginia | 87 | 45 | 45 | 0 | 0 | † | ... | ... |
| Alabama | 79 | 80 | 80 | 9 | 11.2 | † | ... | ... |
| Mississippi | 78 | 80 | 80 | 4 | 5.0 | † | ... | ... |
| South Carolina | 78 | 80 | 80 | 0 | 0 | † | ... | ... |
| South minus Texas | 101 | 2259 | 1289(16) | 160(10) | 12.4 | 970(9) | 626(9) | 64.5 |
| United States minus South | 141 | 5578 | 2444(24) | 288 | 11.8 | 3134(35) | 1547 | 49.4 |
| United States | 129 | 8173 | 3985(40) | 455(36) | 11.4 | 4188(45) | 2210(45) | 53.0 |

* No public-supported medical school.
† No private medical school.
N.B.: numbers in parentheses indicate number of schools if more than one.

Board include the output of one new 4-year school in Texas, the filling of empty third year places in existing schools with students from 2-year basic medical science schools, and increasing the first year enrollment where feasible.

Let's look at the anticipated supply of physicians for Texas. If the current rate of licensing continues without change, we can hope to add the same number of physicians during the next decade as were added during the past decade. In 1949 Texas had 7724 physicians, 6175 were licensed during the decade 1949–1959, and in 1959 Texas had 9625 physicians for a net gain of 1901* (10). Therefore, at the current rate of acquisition we can expect an additional net gain of 1901 from 1959–1969 and one-half that number or 950 from 1969–1974. The resultant total of 12,476 physicians by 1975 must supply an expected population of 13.5 million. *At this rate, the resultant physician/population ratio will drop from 101 to 92 by 1975.*

In 1975, 13,635 physicians will be required to maintain the 1959 Texas ratio of 101/100,000. This is 1159 more than the anticipated 12,476 and is equal to the output of one four-year medical school graduating 116 students each year from 1966 to 1975 (or two four-year medical schools graduating 58 students annually for the same period).

By 1975 we must have 17,415 physicians to raise our ratio to the 1959 average of 129. The net deficit—17,415 minus the projected supply of 12,476—is 4939, very close to the prediction of 5000 rendered by Dr. Godwin of the Southern Regional Education Board on the basis of "high estimates of supply." It seems quixotic to think that Texas could acquire an extra 4939 physicians by 1975, particularly when this would mean putting into operation as many as five new four-year medical schools in the interim. The prospect of obtaining an additional 1159 physicians above the current rate of acquisition in order to maintain our 1959 ratio is far more reasonable and practical.

One of the major reasons that our projected physician supply is inadequate to maintain the present physician/population ratio is the lack of significant increase in the number of matriculants and graduates from Texas medical schools. The rate of output of

* The difference between the gross and net figures represents largely attrition by death, although some emigration of physicians from Texas has occurred.

graduates in Texas has been nearly constant, contrary to the increasing rate in the South and the rest of the United States. Similarly, the rate of licensure in Texas of physicians new to the profession has been constant, also contrary to the tendency in the other areas (Figure 1).

In the period 1949–59 the State of Texas licensed 6175 physicians; 55.5% were new additions to the profession and 45.5% represented physicians who had been licensed in another state and who were ostensibly moving to Texas. In other words, for nearly every physician Texas acquired just out of medical school

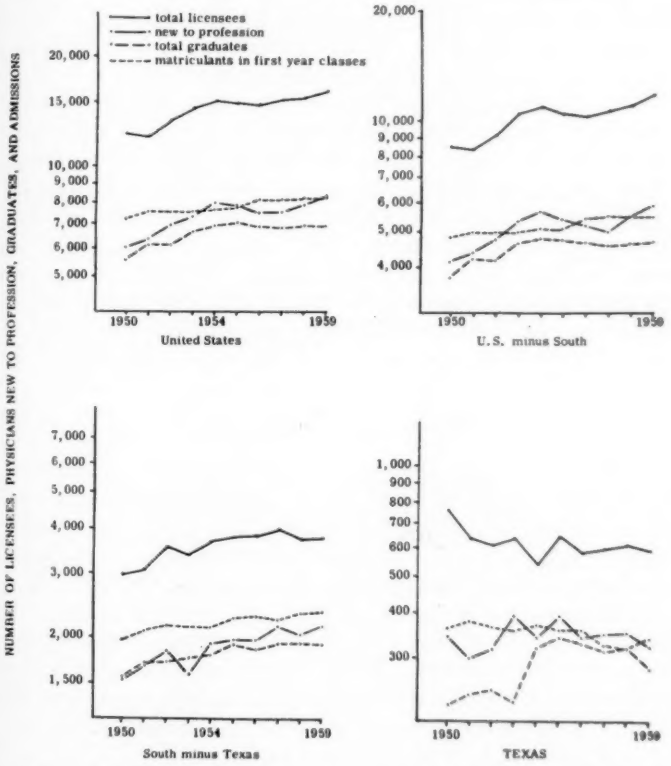


FIG. 1. Comparative rates of several parameters of physician supply (semi-logarithmic graph is used to illustrate rates; if lines are parallel, rates are identical).

(or hospital training) there was one who had come to Texas after practicing elsewhere. If it had not been for these "immigrant" physicians, Texas would have had only 7876 physicians in 1959 and a total physician/population ratio of 72—considerably lower than any other Southern state since 1949. Are we not indebted to other states for graduating physicians that become Texas practitioners? The Texas Commission on Higher Education has recognized this indebtedness and has commented in these words: ". . . in recent years Texas has been educating only about one-half of the physicians admitted to practice in the State each year. Obviously, this is a situation which could not prevail nationally (except in the case of a heavy inflow of foreign-trained doctors), and it indicates that the State is a debtor to the rest of the country in this important respect" (13).

What conclusions can be drawn from these rate data? That there is *no significant increasing source of supply of physicians* for the State of Texas and that the current rate of acquisition of physicians is inadequate to maintain our present physician/population ratio of 101/100,000 (Figure 2)! Wrote the Surgeon General's Consultant Group (14), "If the minimum goal of maintaining present ratios of physicians is to be met, the present medical school facilities must be increased substantially and new schools must be established. This expansion must be undertaken at once. Delay will only magnify the problem."

### *New Medical Schools in Texas*

In House Bill No. 9, 56th Texas Legislature, there is authorization for planning The South Texas Medical School in San Antonio. In October, 1959, a few months later, an Advisory Committee rendered its report which included recommendations for a medical school with an initial entering class of 50 students and a city-county teaching hospital of 500 beds (5). On January 31, 1961, the voters of Bexar County overwhelmingly supported the prospective new San Antonio medical school by passing a $6.5 million dollar hospital bond issue by a 6.7 to 1 majority; the biggest majority ever accorded a bond issue in Bexar County (12).

That the South Texas Medical School will be a reality is almost certain. Whether it will materialize early enough to make a significant difference in the availability of physicians during

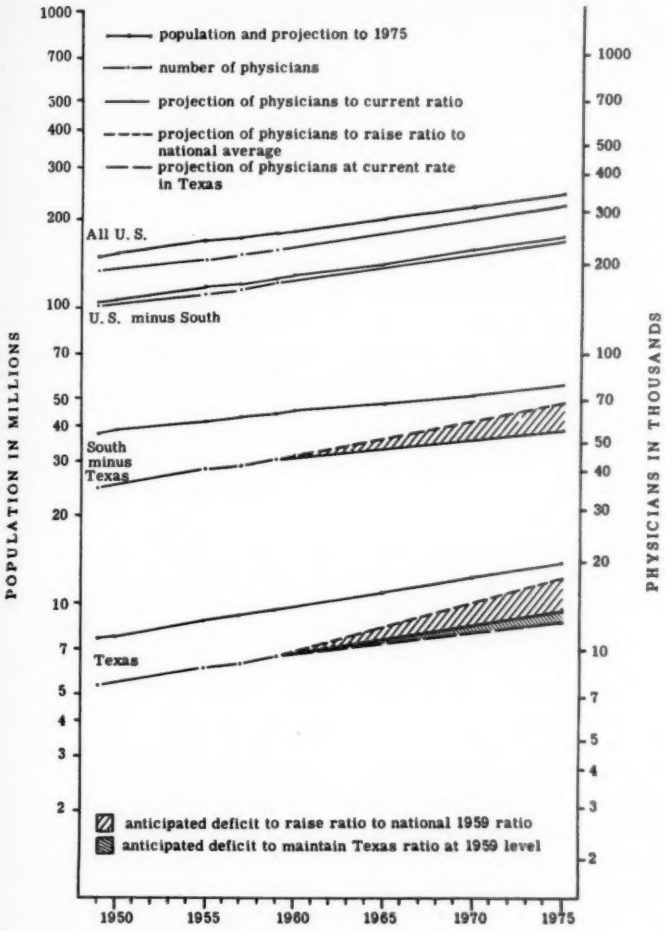


FIG. 2. Population and physician projections.

the critical years before 1975 is difficult to evaluate at the present time. It is commonly estimated that it takes about 7 to 14 years to turn out the first graduates after the first appropriations are made.

Flexner, in his report of 1910 (7) and its sequel of 1925 (6),

pleaded mightily for the incorporation of medical schools into the fold of the university, not just in name, but *in toto*, so that the medical faculty and curriculum and students would be as much a part of the university as the other teachers and students, thereby preserving the requisite scholarly atmosphere, for it is difficult to be "Enflamed with the study of learning . . ." elsewhere (11).

Of the present three medical schools in Texas, not one is less than 150 miles from its parent university and the proposed fourth school will be more than 70 miles away. Not until Texas obtains a medical school on the same campus (or at least in the same city) as the parent university will Flexner's entreaties and those of many others be fulfilled. In actual practice, relationships of medical schools to their parent universities have tended to be remote. Few have had mutually rewarding associations with other graduate departments. Nonetheless, the physical proximity of the medical school and its university seems to be of expanding value as medicine becomes more and more involved in research in general, and in physical and social science research in particular (14).

Concurrent with the development of the graduate program at the University of Texas along the lines recommended by the Committee of 75 (4)—"the graduate program should provide a framework within which topflight professional education may take place"—it seems that a medical school in Austin would probably provide a more fertile milieu for high academic attainment than any of the present or proposed locations. In addition, Austin would probably be the most likely location for a 2-year basic medical science faculty that could be established on a much more rapid schedule than an entirely new institutional branch in San Antonio. Such a 2-year basic medical science school in Austin, if established on a more-or-less crash program, could provide students to fill the third year places emptied by attrition in the present three four-year medical schools in Texas as well as some third year transfers to out-of-state medical schools before the completion of the South Texas Medical School.

Since World War II five of the eight basic medical science schools in the United States have expanded into four-year programs (10), thus reducing the available students to fill empty third year places resulting from attrition of the first two years.

Not a single new basic medical science school has been established during these last 15 years.

The 1958 Report of the American Medical Association Council on Medical Education and Hospitals (1) urged the development of several new schools of the basic medical sciences in hopes that as many as 300 medical graduates could be added annually. In the opinion of the Council, basic medical science schools can be obtained at much less cost because of the lower capital and operating expenses of two-year programs. The Council further stated that two-year programs should offer a closer and more meaningful relationship between liberal education and early medical education; the faculty and facilities of the basic medical sciences would add breadth and depth in biological disciplines important to the overall university programs.

This intimate relationship would offer fertile ground for curricular innovations, melding the upper division college training with the basic medical sciences and melding graduate non-medical training with the basic medical sciences. Cross-stimulation of this nature would assist immeasurably in preventing the continued siphoning of able, scientifically-minded students away from biological interests. Therefore it seems quite likely that a two-year basic science medical school in Austin would attract students of high quality and provide an impetus to the development of the science of medicine in Texas, as well as lending a more immediate partial remedy to the problem of physician supply in Texas.

### *Supply of Qualified Students*

The birth of more medical schools in Texas will raise a commanding question: *Where will additional properly qualified students be obtained?*

At the present time, almost every Texan with minimum qualifications can obtain an acceptance letter. (In the opinion of the author, some Texans with qualifications *below* the minimum standards are admitted to Texas medical schools in preference to non-residents with qualifications substantially more than minimal). Because of the lack of properly qualified applicants, one Texas medical school has been forced to reduce the size of its entering classes for the past several years.

There is little likelihood that the development of more medical schools in Texas can be successfully executed within the next few years without a concurrent major revision in residence requirements. If such revision is not forthcoming, either there will not be qualified candiates for the increased enrollment or the additional students will come from the remaining applicants *with below minimum qualifications.**

It appears that there is some direct relationship between the availability of physicians in a state and the willingness of the medical schools, particularly the public-supported schools, in that state to admit a significant number of non-residents to the first year classes. In neither of the two public-supported Texas medical schools is more than a token number of non-resident students accepted.

All but one of the ten private medical schools in the South admit high percentages of non-resident students. In the six Southern states with physician/population ratios higher than Texas' there are six public-supported medical schools which admit a collective total of 21.4% non-residents. In the nine Southern states having a lower physician/population ratio than Texas' there are eight public-supported medical schools which admit a corresponding collective total of 3.5% non-residents (Tables 1 and 2).

A recent tabulation by the Southern Regional Education Board revealed that public-supported medical schools in the South admitted students with averages on the Medical College Admission Test which were the lowest of all geographical areas. The Southern private medical schools, on the other hand, admitted students with MCAT averages very close to the national average of all accepted students. This difference in average Medical College Admission Test scores is apparent in comparing most schools with unduly rigid residence requirements to other schools with liberal non-resident policies. Attrition rates also exhibit similar differences—the highest attrition rates, as a rule, are found in those public-supported medical schools with strict residence requirements (and sub-standard budgets) (2, 8).

* Minimum qualifications include moral, ethical, health, personal, and scholastic standards as well as other pertinent characteristics of the applicant; the possession of a requisite grade point average does not *per se* indicate that the student meets the minimum qualifications for admission to medical school.

TABLE 2

*Composite totals and percentages comparing Texas to those states in the South having physician/population ratios above and below that of Texas, 1959*

| | Public-supported medical schools | | | Private medical schools | | |
|---|---|---|---|---|---|---|
| | Total admissions | Non-resident admissions | Per cent non-residents | Total admissions | Non-resident admissions | Per cent non-residents |
| States having a physician/population ratio higher than Texas | 640(6) | 137(5) | 21.4 | 343(5) | 219(5) | 60.4 |
| TEXAS | 252(2) | 7(2) | 2.8 | 84 | 37 | 44.0 |
| States having a physician/population ratio lower than Texas | 649(8) | 23(5) | 3.5 | 307(4) | 132(4) | 40.3 |

N.B.: numbers in parentheses indicate number of schools if more than one.

These two differences, as measured by average scores on the MCAT and attrition rates, are major indices of academic endeavor and reflect, therefore, the higher attainment in medical schools with greater competition for admission. The remaining schools, those that have artificial barriers to the selection of the best qualified candidates, have considerably less competition and a correspondingly lower level of academic attainment.

Equally important in the potential supply of qualified applicants for Texas medical schools is the apparent lack of full recognition and more adequate development of Texas' own native talent. This feature is so important that it will be the subject of a subsequent article.

The choice seems clear—either Texas will recognize the magnitude of the problem and take steps to rectify each portion of it, or she will not. A quotation from a speech given by the former Vice-President for Medical Affairs, The University of Texas, is applicable: "Despite the fact that the quality of graduates from medical schools must never be compromised by demands for quantity, educators, now or in the future, cannot disregard their responsibility to produce an adequate supply of physicians" (3).

## *Summary*

1. By 1975 1159 more physicians than the current rate of supply can be expected to provide will be needed to maintain our

1959 physician/population ratio of 101/100,000; otherwise, our ratio will possibly drop as low as 92/100,000.

2. Texas cannot continue to expect the other states to supply half of Texas' needs for physicians.

3. A new source of physicians is necessary sooner than the South Texas Medical School can provide them, and a greater total supply than the present medical schools *plus* the South Texas Medical School can provide is mandatory.

4. A two-year basic medical science school in Austin is recommended for practical as well as academic reasons.

5. Texas must admit a much higher percentage of nonresident students to her medical schools in order to raise the academic level as well as to help relieve the shortage of physicians.

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# THE EXPANDING HORIZON IN NUTRITION[a]

ROGER J. WILLIAMS

In almost any area of science which undergoes intensive study, changing points of view are likely to emerge bringing new vistas of unanswered questions. The science of nutrition is no exception; it is ready for a change in outlook which can almost amount to a metamorphosis. In the earlier stages of the development of the science of nutrition, progress consisted primarily of finding out more about the chemistry of nutrition in various species of experimental animals and ultimately the human species, on the assumption that each species constitutes a discrete unit, which must be explored by itself and in its relation to other units.

This approach has been very productive in spite of limitations which appear now that many basic nutritional items have been recognized and catalogued. These limitations must cause a modification of the concept that each species is a unit, because findings on the borderline between biochemistry and genetics make it apparent that each breed, race and strain which is genetically different must possess corresponding differences with respect to nutrition and that individuals within each strain who possess genetic dissimilarities must also possess corresponding dissimilarities in their nutrition. How important these dissimilarities may be, will be suggested in later paragraphs.

Furthermore even the concept that an individual animal is a unit, so far as nutritional study is concerned, must not be adhered to too closely. An individual animal may be looked upon as a coordinated set of many kinds of cells with different sets of nutritional needs, all in competition with each other for material carried by the blood stream. It might be very desirable for us to know about the distinctive needs of the different cells and tissues and this might constitute one objective of tissue culture studies.

The writer's first bout with problems of nutrition had to do with the nutrition of single yeast cells growing in media of known

[a] Contribution from the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas, Austin. Accepted for publication January 1961.

composition in hanging droplets (19). This experience in the field of yeast nutrition has conditioned me to view the problems of nutrition from the cellular point of view.

In nutritional studies with yeast it became apparent, relatively early, that different strains of yeast belonging to the "species" *Saccharomyces cerevisiae*, behaved entirely differently (20); not only this but individual yeast cells showed wide variation in their response to particular nutritional regimens. This was observed long before the existence of yeast genetics was recognized.

One of the most striking findings which took many years to clarify and establish in the writer's mind, was that for yeast cells "optimal" nutrition is practically an impossibility. If one should concoct what he thought was a perfectly balanced "optimal" medium for yeast growth, he might find that a slight change in the environment such as a rise or lowering of temperature or pH, would throw the medium out of balance and that much juggling of the constituents would be required before the medium was again close to "optimal".

Very early in the writer's experience it was observed that amino acids introduced into a synthetic culture medium would often depress yeast growth (19), and it was concluded at the time that these nutrients were deleterious to yeast growth. Many years later, after the identity of various vitamins involved in yeast nutrition was established, a repetition of the amino acid experiments was made using basal media well fortified with the necessary vitamins, and the results were diametrically opposite (12). The vitamin ingredients had become so well adjusted that now amino acids had become limiting factors for growth, and their introduction into the culture medium caused large increases in growth. It seems likely on the basis of extensive experience that after improving the culture medium by the addition of amino acids, it would have been necessary to readjust all the vitamin levels, if an "optimal" medium was sought. This balancing of each ingredient against all the others could go on almost *ad infinitum* without arriving at any medium which would be truly optimal.

Another complication arises when we ask the question "Optimal for what?—Optimal for maximum growth, for producing yeast with high fermenting power, for producing yeast with good keeping qualities, for producing yeast with relatively large cells,

or what?" The optimal medium (or conditions) would not be the same if judged by different criteria.

It is the writer's opinion that a parallel situation exists with respect to every cell and tissue in our bodies. There is probably no cell in the body of a healthy person the nutrition of which could not be "improved", for example in the sense of being made more active metabolically, by a modified balance between the various minerals, amino acids, vitamins and other nutrients that are supplied by the blood stream. It is by no means certain, however, that it is desirable for every cell in our bodies to be working continuously at maximum capacity.

One of the questions which immediately arises in connection with this discussion is a very fundamental one: whether cells acquire, during the process of differentiation, at least quantitatively different nutritional needs. There can be, it seems, only one answer to this question—an affirmative one. To take an extreme example it seems incontestable that the differentiated cells which produce the thyroid hormone have a need for relatively large quantities of iodine—far out of line from the iodine requirement of all other cells. It seems likely that the cells of the islets of Langerhans, for example, have nutritional needs which are geared to the production of insulin, and since insulin contains 12% cystine, several times the average for other proteins, it seems inescapable that sulfur containing amino acids would be required in relatively large amounts by insulin-producing cells. The cells in the pituitary which produce the growth hormone or the lactogenic hormone, or the thyreotropic hormone, must be furnished suitable amounts of the requisite amino acids. The cells in the adrenal cortex which produce the adrenal hormones, must require the peculiar raw materials required for the production of these steroid hormones, the muscle cells must need the raw materials for building and maintaining muscle, the bone marrow cells must be furnished those nutritionally essential amino acids contained in hemoglobin, and the nerve cells (the composition of which is very different from that of many other cells) must have nutritional requirements geared to the maintenance of nerve cells.

With this concept and our previous discussion in mind it seems clear that even under conditions of maximum total health there is competition between the cells and tissues of the organism for

the available nutritional factors. Any type of cell or tissue has the potentiality of being rendered metabolically more active by improving its nutritional environment, *i.e.*, by supplying its peculiar needs in balanced and abundant quantities. In maximum health, the thyroid hormone-producing cells, the insulin-producing cells and all the pituitary cells, etc., etc., are manufacturing their products and carrying on their activities at a satisfactory rate and it would not be desirable for this balance to be upset by providing the thyroid-producing cells, for example, with a nutritional environment which would cause a doubling of the output of thyroid hormone.

This same concept, however, leads us to another conclusion which has important practical potentialities, namely that if there is lack of maximum health and an imbalance of activities, inadequacies of cellular nutrition may be a causative factor. This is actually the case in endemic goiter.

In regions where simple goiter is prevalent, there are many individuals who are not afflicted. Their thyroid glands are highly efficient in retaining and making use of the iodine that is available; on top of this their needs for thyroid hormone may be relatively low. There are others, however, who cannot pick up enough iodine, and as a result are hypothyroid. If such individuals are furnished iodized salt, for example, the nutritional environment of the thyroid gland is improved and the hypothyroidism vanishes. Even in regions where simple goiter is not endemic, there are individuals whose thyroids are hypoactive by reason of a lack of sufficient nutrient iodine to supply their relatively high needs.

Though no practical use has been made of the fact so far as I know, the production of insulin may be, in some cases, limited by the inadequacy of the nutritional environment of the insulin-producing cells. It seems likely that in a percentage of cases of hypoinsulinism, the condition might be materially improved by supplementing the diet with an abundance of sulfur containing amino acids. Unless the results were of a striking nature, this relatively expensive and bothersome (to the physician) approach would not be a popular one.

There are basic reasons besides lack of abundant nutrition, however, which must often be highly important in causing a specific tissue or a gland to be under-active. The sizes of the

glands or the amount of active tissue present must be highly important limiting factors. Thyroid glands in normal individuals vary in weight from 8–50 grams (6), adrenal cortices are said to vary 10-fold in thickness (5), the number of islets of Langerhans in the normal pancreas varies from about 200,000 up to 2,500,000 (13).

It seems reasonable to suppose that if one has a minimum sized thyroid or adrenal cortex or a minimum number of islets of Langerhans, that improving the nutritional environment of these quantitatively deficient tissues would be most desirable. On the other hand, however, if one were endowed with an extra large thyroid gland, an extra thick adrenal cortex or a very large number of islets of Langerhans, improvement of the nutritional environment for the abundant specific cells and tissues would be contraindicated.

As is well known, every functioning human body is a coordinated system. The checks and balances and the regulating mechanisms which control the size and activities of various organs and tissues are largely unknown. Circulatory changes probably play an important role. It seems probable in view of our discussion that homeostatic withholding of optimal nutrition by competitive measures may be one mechanism whereby the activity of specific superabundant cells may be held in check when the health of other cells demands it. It may likewise be that there are mechanisms for favoring, nutritionally, those cells and tissues which are deficient quantitatively. In any case, the potentiality exists to improve the nutritional environment of any specific cells and tissues known to be limited anatomically, by furnishing the body as a whole with an extra supply of the special nutrients (if they exist and are known) needed by these special tissues.

If one adopts the point of view of cellular and tissue nutrition, one is confronted with certain questions which it seems profitable to ask. For example, do all the cells and tissues have the same synthetic abilities, or would the list of "essential" amino acids, for example, be different for each tissue? In case only essential amino acids were fed, would some tissues have to depend upon others for a supply of certain non-essential amino acids? Can a perfectly definite line be drawn between "essential" and "non-essential" amino acids or are there some so-called non-essential

ones the production of which may be for various reasons serious limiting factors?

That the differences between the synthetic abilities of cells and tissues enter in an important way into the problem of the nutrition of the body as a whole, and that "essentiality" is not as definite as might be supposed is indicated by several facts, some of which have long been recognized. (a) Certain cells in the thyroid, it is recognized, are the only ones capable of synthesizing the amino acids triiodothyronine and thyroxin. (b) When a nutrient such as riboflavin, for example, which is thought to be needed by every cell and tissue in the body, is lacking in the diet of an individual, *local* lesions result, indicating that the riboflavin requirement of the affected tissues is unusually high or its local presence unusually important. (c) Vitamin A, presumed not to be needed by all tissues, is apparently needed in different localities in widely different amounts, because the requirements of animals as judged by different criteria, vary at least 20-fold (22). (d) A microbiological study of the vitamin B contents of various tissues has shown clearly that each tissue has a distinctive profile (18) suggesting that the metabolism in each is distinctive. (e) From diverse investigations and general experience it is clear that the enzyme levels in different tissues are distinctive, again indicating substantial differences in metabolism. This is a strong indication that the nutritional needs of individual cells and tissues are likewise distinctive, because one of the most crucial functions of nutrition is to supply the raw materials for enzyme production. (f) A complete assortment of amino acids when fed to animals is superior nutritionally to one containing only the essential amino acids (1). (g) Arginine has often been regarded as non-essential for rat nutrition, yet it may be a limiting factor for growth of young (15). (h) Recent tissue culture studies show that cultures from liver, conjunctiva and intestine require for growth in addition to recognized essential amino acids, arginine, cystine, glutamine, and histidine, which are not regarded as "essential" (4). Tryptophane may be only stimulatory in some cases. (i) Arginine in humans appears to be a local limiting factor in spermatogenesis (7, 1). (j) Glutamine though classed as a non-essential amino acid appears to be in a substantial proportion of the population a limiting nutritional factor for health under present prevailing conditions (14, 17). (k) The quantitative

needs for individual amino acids, vary from individual to individual widely and to such an extent as to suggest that differences in organ and tissue needs are involved (23).

The elegant results obtained by Rose and others using maintenance of nitrogen balance in young men and young women as the criterion for essentiality of specific amino acids, need not be interpreted rigidly. Under the conditions of the experiment the contrast between "essential" and "non-essential" amino acids stand out, yet some of the "non-essential" amino acids may be synthesized only in certain tissues and may be "essential" so far as other tissues are concerned. Glutamine, for example, must be produced somewhere in the body because nitrogen equilibrium can be maintained without its being supplied nutritionally; yet it appears "essential" for certain cells in liver, intestine and conjunctiva. These tissues must be dependent upon other tissues in the body for their glutamine supply. If these other tissues have for one reason or another a limited ability to produce glutamine (beyond their own needs) then the glutamine may become an "essential" amino acid for the body as a whole. This might not be expected to show up in a short term experiment or to be uniform for different individuals.

Especially would a borderline amino acid appear "non-essential" if the manufacturing tissue were relatively large like the liver and the consuming dependent tissue were small like the pituitary gland, for example. In such a case it might be very rare that a liver could not produce enough of the amino acid in question to meet its needs and those of the pituitary also, yet in some cases because of a partial genetic block, or an unusually small liver (normal livers vary about 5-fold in size) or both, the supply might be too limited. There could also be a partial genetic block on the other end of the system, resulting in the pituitary gland not being able to utilize very low concentrations of the needed amino acid.

If we accept the point of view that cells and tissues vary from cell to cell and tissue to tissue in their amino acid needs and in their synthetic abilities, and if we recognize the tremendous variation in organ weights in normal individuals (the median range is 10-fold for male rabbits of the same breed, even after correcting for differences in body weight) (2) we would be prepared to accept the fact, determined in actual experiments, that

the normal variation in the human needs for lysine, methionine, threonine and leucine are from 4 to 6.5-fold (23). These wide variations have been exhibited in very small groups of individuals and would doubtless be of greater magnitude if a large population were studied. According to the classical concept of nutrition everyone who is normal has normal nutritional needs and every observed variation of substantial proportions is a source of embarrassment and tends to be disregarded or neglected because it does not fall within the purview of the orderly scientific facts. There is no room in the classical concept for the fact that the calcium needs of individuals certainly vary 5-fold and probably more, accompanied by a 5.7-fold variation (among young men) in bone density as determined by X-rays. The strong evidence that the needs of "normal" human beings for vitamin A, ascorbic acid, vitamin D, several of the B vitamins, and for the amino acids methionine, threonine and leucine vary *not less* than fivefold, is something that is incomprehensible to one who regards "*the* human being" as one whose nutritional needs can be determined with certainty and tabulated with assurance. According to current opinion a physically active man needs daily about 70 grams of protein, 1 gram of calcium, 12. mg. of iron, 5,000 units of vitamin A, 1.5 mg. of thiamin, 1.8 mg. of riboflavin, 15. mg. of niacin and 75. mg. of ascorbic acid. These estimates do not allow for all the existing several-fold variations, which, however, are completely comprehensible if we take into account cellular nutrition, variation in organ sizes, and modern knowledge about partial genetic blocks.

It appears likely also that vitamin requirements do not always hold as rigidly as has been supposed. Lipoic acid may be a case in point. It is undoubtedly *used* by many different types of cells in animal organisms; unlike other recognized vitamins its endogenous production in mammals seems to take place. There are reasons for thinking, however, that it may occupy a position similar to arginine and glutamine among the amino acids; it seems probable that it is a nutritional essential for *some* cells and tissues; these particular cells and tissues are hence dependent for their supply on other tissues. On this basis, taking cellular nutrition into account, it is possible to explain the experimental findings in various laboratories.

In our laboratories clear-cut highly significant vitamin effects

were noted both in rats (Wistar) and chicks (Leghorns), when minute amounts of lipoic acid were added to synthetic diets (3). Subsequent repetition of these experiments on chicks in our laboratories have failed to confirm the original findings. In the du Pont laboratories, similar contradictory results have been obtained, sometimes lipoic acid has yielded statistically significant effects with chicks, at other times the effects have clearly been without statistical significance (16). The consensus among nutritionists, based in part on experiments which have not been reported in full, is that rats do not require lipoic acid. The possible differences in strains, however, have not been explored. Mardones *et al.* (11) have found that lipoic acid is a vitamin-like factor which decreases voluntary alcohol consumption in rats. We have not been able to reproduce their results with our rat strains.

In the light of our discussion it seems most plausible that some tissues (only) within the chicks and rats require lipoic acid, and that the supply from other tissues (which are often capable of producing an excess beyond their own needs) is somewhat uncertain. In some cases, for genetic (and possibly other unrecognized reasons) the issues which require lipoic acid do not get enough and are hence helped by an exogenous supply. Among the genetic factors which might be operative are partial genetic blocks which might limit production of lipoic acid on the one hand or its utilization at low concentrations on the other.

Inositol may be another case in point. Isotopic studies indicate that it can be synthesized in the tissues of higher animals (2a, 6a), yet it is needed by human bone marrow and liver cells in tissue culture (4a), and nutritional deficiencies have been produced in five different mammals and two fowls.

One of the interesting questions which arises in view of the wide variations in the nutritional needs of individuals, pertains to the importance of self selection in satisfying these needs. A judicious consideration of this problem must avoid, it seems, two conflicting extreme views. One is that self selection works perfectly, that everyone will, if given an opportunity, eat what he needs. If this were true the study of nutrition would be of little or no practical value. The other extreme is the view that appetites are completely untrustworthy; no one has any innate sense to choose wisely.

Somewhere between these views lies the truth, and as we shall attempt to make clear, the intermediate position of the truth is not the same for different individuals. We know, for example, that the appetite mechanisms which help govern how much one eats are vastly superior in some individuals than in others. Some go throughout life depending practically entirely on their "body wisdom" to tell them how much or how little to eat, with the result that they are always well fed but not overfed. Others on the other hand, if they depended on body wisdom alone would become highly obese.

Specific appetites differ also in a similar manner. Some individuals have an excessively "sweet tooth" and because of this tend to eat excess quantities of sugar. Other individuals on the other hand lack a desire for sweets and do not fall into this nutritional error.

A most significant finding from the standpoint of cellular nutrition, was first made by Macy (10) with respect to children and later confirmed with experimental animals (21), namely that improved nutrition decreases the appetite for sugar. This fact strongly suggests that the appetite center itself is capable of being well-nourished or malnourished, and that good nutrition fosters "body wisdom" (avoiding excess sugar) and that malnutrition of the appetite center promotes "body foolishness" (the consumption of excess sugar).

It would appear then that good nutrition fosters good nutrition, and it is reasonable to suppose that the nutrition of the nerve cells in the appetite center would be one factor which would help determine whether or not an individual has body wisdom with respect to his general and specific appetites.

No one can deny the possibility that the individual who has a strong tendency to become obese, is suffering from a malnourished appetite center. We do not know what the nutritional needs of the cells which constitute the appetite center are, nor are we certain that we could supply these needs (if known) to the proper area. In view of the enormous importance of the problem of obesity psychologically and health-wise, this is an approach which is worth considering.

Alcoholism is a disease which involves a derangement of appetite, in that afflicted individuals have an uncontrollable urge to drink. Looking at this problem from the standpoint of cellular

nutrition and metabolism leads one to suspect that alcohol has for genetic reasons an unusual ability to poison the appetite controlling cells in certain individuals, but that being well nourished increases the resistance of these cells to this poisoning. A great deal of evidence involving both experimental animals and human beings has been accumulated to support this hypothesis (24). Experiments have been carried out so many times with concordant results that it can be stated unequivocally that in experimental animals abundant nutrition (of the whole animal) greatly decreases the voluntary consumption of alcohol.

Up to this point we have discussed for the most part the scientific rather than the practical or applied phases of nutrition. Admittedly the introduction of the point of view which we have developed may greatly disturb the thinking of many who are accustomed to deal with averages and seek on a population-wide basis to improve nutrition. In these situations practicalities must be considered as a matter of course, however, and it is obviously impractical to determine the distinctive needs of everyone in a population. It is not impractical, however, in the foreseeable future to establish more definitely than is now known how great variations to expect. When it comes to a consideration of the eradication of a specific recognized deficiency disease, practical experience alone will show what measures are effective; if these measures work, the prime question under consideration has already been answered.

It is the writer's opinion that the facts of nutritional variability are such that nutrition will still be a live subject for a thousand years at least. If this and other forms of variability did not exist in the human species, nutrition (and life in general) might become crystallized, and rules could be established to such an extent that it would be no longer necessary to use intelligence. This outcome does not, in view of the facts we have been considering, appear to be imminent.

Future applications of the science of nutrition will depend to a considerable degree, in the writer's opinion, on a better understanding of the facts of cellular and tissue nutrition, and may encompass almost every type of known pathology including deformities and damaging morphological aberrations.

The importance of nutritional considerations in connection with therapies in general will increase. A few examples may

serve to indicate some of the directions in which advances may be expected. For example, it is clear that numerous types of hormone deficiencies must exist. If these are duly recognized, the administration of the hormone in question immediately suggests itself as an appropriate therapy. A consideration of cellular nutrition leads one to think, however, of the possibility of improving the nutritional environment of the hormone producing cells so that they can produce and administer at the natural site, the hormone which is in short supply. This has not been tried in any specific case so far as I know, but it is possible that nutritional therapy may some day be highly valuable in the many pathologies in which hormone deficiencies must play a role.

In medical thought a differentiation must be made between nutritional therapeutic agents which are constructive, on the one hand, and the anti-agents of various sorts, on the other, which have been found, often empirically, to be useful in the treatment of disease. A few years ago observations were made in our laboratory indicating that pantothenic acid was an agent which could be used to combat allergy. When this idea was submitted to a pharmaceutical house for trial, however, it was found by the practically minded testers, that while pantothenic acid was somewhat effective it was not superior to certain available antihistaminics!

In another connection, it has been noted that pantothenic acid may be an effective agent for combating constipation. Individuals have reported its use and indispensability over a period of years for this purpose. If it works as indicated, it does so by strengthening and improving the metabolism of the cells and tissues involved in peristalsis; this is entirely different from a laxative which is often effective because it is noxious. The two types of therapy should not be in competition with each other, but insofar as anything is known by physicians or the public about the use of pantothenic acid for this purpose, there is direct competition, and of course the convenient, advertised, and more certain laxatives win out.

Paralytic ileus, which is very commonly responsible for severe pain and discomfort after abdominal surgery has been found to be treated with signal success by pantothenic acid (8). Prostigmin, a drug which inactivates the enzyme cholinesterase, has often been used for this purpose. The two agents must act in

entirely different ways but it is a common error to consider them as direct competitors.

In the course of his experience with alcoholics, the author has found many examples in which a balanced nutritional supplement greatly enhanced an individual's ability to sleep. Non-alcoholics have also reported the same observation. If this supplement works as indicated, it does so presumably by improving the nourishment of nerve cells which are involved in normal sleep. This nutritional therapy for the condition of insomnia should not, in order to be used, have to compete with barbiturates and other sleep-producing drugs which are certainly not constructive in their action.

William Kaufman (9), a physician, has carefully worked out objective quantitative measurements for joint dysfunction and has found that relatively large doses of niacinamide invariably improve the functioning of the joints of arthritics. This nutritional approach is not taken seriously by most physicians, and niacinamide cannot compete unassisted with the remedial use of salicylate drugs, for example, which obviously again are not constructive in their action.

Gradually there will be, as a result of consideration of cellular and tissue nutrition, an abandonment of the earlier point of view, still held in some circles, that if one escapes beriberi, scurvy, pellagra, rickets and the recognized deficiency diseases, his nutrition is above criticism and further improvement is impossible. Nutritional therapy will in all probability be found indispensable, when we know more than at present, in the treatment of hundreds of diseases.

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# VARIATION IN NUCLEIC ACID PHOSPHORUS CONTENT BETWEEN STRAINS OF THE SAME SPECIES OF BACILLUS[a]

E. O. BENNETT[b] AND R. P. WILLIAMS

Since the publication by Schmidt and Thannhauser (1945) and by Schneider (1945) of methods for the estimation of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content of tissues, many reports have appeared concerning the nucleic acid content of various bacterial species. Davidson (1953) and Leslie (1955) have summarized several of these investigations. In general the determinations were carried out on one strain of a given species. Winder and Denney (1956) examined various strains of *Mycobacterium phlei* and *Mycobacterium tuberculosis* for their DNA and RNA content, but the growth conditions of the organisms were varied during the study. Mitchell and Moyle (1954) investigated the DNA and RNA content of several Gram positive and negative bacteria, and by means of histograms gave an indication of the variation from the arithmetic mean of individual determinations carried out on separate species. However, no study was made of the variation in content that might occur within several strains of the same species of microorganism.

In view of the large number of papers being published reporting the DNA and RNA content of bacterial species, it seemed pertinent to investigate the variation in DNA and RNA content that might occur between several strains of the same species of bacterium when they are examined under identical growth conditions. The data presented in this paper have been collected from determinations carried out on the vegetative cells of 33 strains of *Bacillus anthracis* and 10 strains of *Bacillus cereus*.

## *Materials and Methods*

The cultures employed were obtained from the collection of Dr. Kenneth L. Burdon or from the stock collection of the University of Houston. Each

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culture was identified as to the correct genus and species designation. The organisms were maintained on a beef-peptone-yeast (BPY) agar composed of beef extract (Armour) 3 gm., peptone (Difco) 5 gm., yeast extract (Difco) 1 gm., and agar (Difco) 20 gm. in 1-liter of distilled water. The medium was adjusted to pH 7.0 and autoclaved at 15 lbs. pressure for 20 minutes.

Before analysis each strain was streaked on BPY agar plates to determine the purity of the culture. An isolated colony was selected from the plate culture, and transferred to a BPY agar slant. A series of daily transfers of each culture were then made on BPY agar slants to stabilize the strain. The inoculum was prepared from a BPY agar slant culture incubated for 24 hrs. at 37° C. The growth from this slant was suspended in 0.9 per cent saline, and 1.0 ml. of the material used to inoculate the surface of a Kolle flask containing BPY agar. The Kolle flasks were incubated at 37° C. for 18 hrs. Microscopic examination of the individual cultures established that the growth at this time consisted of vegetative cells. The growth was washed off the agar surfaces with 0.9 per cent NaCl. The washings were transferred to a 50 ml. centrifuge tube, and the cells collected by centrifugation. The harvested cells were washed two times by centrifugation in saline, and then suspended in 4.5 ml. of the saline solution. A 1.0 ml. sample of this suspension was dried at 150° C. to constant weight to determine the dry weight of cells. Other 1.0 ml. samples were employed for DNA and RNA determinations. All analyses were carried out on duplicate samples of organisms harvested from ten different Kolle flask cultures of the same strain. The values reported are the average of the duplicate set of ten determinations and thus represent the average of twenty different determinations carried out on the same strain.

The nucleic acid fractionations were carried out according to the method of Schmidt and Thannhauser (1945). The 1.0 ml. sample of cells was extracted in the cold with an equal volume of 10 per cent trichloroacetic acid. The material was centrifuged in the cold, and the precipitate extracted with an equal volume of cold 5 per cent trichloroacetic acid. The precipitate was centrifuged, and the supernatant discarded. The residue was treated once with 95 per cent ethanol, twice with an ethanol: ethyl ether (3:1) mixture, and once with ethyl ether alone. The residue remaining after each treatment was removed by centrifugation, and the supernatant discarded. The precipitate remaining after the last ethyl ether treatment was then extracted for 12 hrs. at 37° C. with 1.0 ml. of 1 *N* KOH. The alkaline digested material was acidified with 0.5 ml of 6 *N* HCl, and 3.0 ml. of 10 per cent trichloroacetic acid were added. The ensuing precipitate was separated by centrifugation, and DNA phosphorus determinations were carried out on the precipitate after washing it once with 1.0 ml. of 10 per cent trichloroacetic acid. The supernatant material was employed for the estimation of RNA phosphorus. The amount of phosphorus in the samples was determined by the colorimetric method of Fiske and SubbaRow (1925) using a Klett-Summerson colorimeter with a 660 $m\mu$. filter. The results are expressed as $\mu g$. of phosphorus per mg. of dry cells.

Statistical calculations were carried out on the data employing accepted formulas (Snedecor, 1946). The duplicate determinations of each sample were first averaged, and then the mean of these ten values was obtained. This mean

is the figure reported in the tables. The mean of these figures was calculated by averaging the values, and the standard error of the mean then determined.

## *Results*

The nucleic acid phosphorus values and the RNA to DNA ratios obtained from the analyses of 33 different strains of *B. anthracis* are presented in Table 1. The DNA phosphorus content

TABLE 1

*Nucleic acid phosphorus content of Bacillus anthracis strains*

| Strain number | DNA* | RNA* | RNA to DNA |
|---|---|---|---|
| Ba1(1)a | 0.46 | 3.47 | 7.5 |
| Ba1(2)a | 0.44 | 2.72 | 6.2 |
| Ba1(3)a | 0.63 | 3.66 | 5.8 |
| Ba1(4)a | 0.62 | 3.76 | 6.1 |
| Ba1(6)a | 0.62 | 2.87 | 4.6 |
| Ba1(7)v | 0.82 | 3.30 | 4.0 |
| Ba1(9)a | 0.53 | 2.32 | 4.4 |
| Ba1(10)a | 0.46 | 4.13 | 9.0 |
| Ba1(11)a | 0.46 | 2.67 | 5.8 |
| Ba1(12)a | 0.59 | 3.53 | 6.0 |
| Ba1(13)a | 0.56 | 4.06 | 7.3 |
| Ba1(14)a | 0.39 | 2.94 | 7.5 |
| Ba1(15)a | 0.50 | 3.28 | 6.6 |
| Ba1(16)a | 0.65 | 4.13 | 6.4 |
| Ba1(17)a | 0.62 | 3.76 | 6.1 |
| Ba1(18)a | 0.48 | 2.87 | 6.0 |
| Ba1(19)v | 0.96 | 4.45 | 4.5 |
| Ba1(20)v | 0.80 | 4.13 | 5.2 |
| Ba1(23)v | 0.96 | 4.45 | 4.5 |
| Ba1(24)v | 0.78 | 3.12 | 4.0 |
| Ba1(26)v | 0.74 | 3.85 | 5.2 |
| Ba1(27)v | 1.01 | 2.66 | 2.6 |
| Ba1(28)v | 0.74 | 3.50 | 4.7 |
| Ba1(38)v | 0.62 | 2.46 | 4.0 |
| Ba1(40)v | 0.69 | 3.81 | 5.5 |
| Ba1(43)v | 0.90 | 3.21 | 3.6 |
| Ba1(46)v | 0.84 | 4.13 | 4.9 |
| Ba1(47)v | 0.76 | 3.32 | 4.4 |
| Ba1(52)v | 0.88 | 3.92 | 4.5 |
| Ba1(54)v | 0.79 | 4.07 | 5.2 |
| BUCM1v | 0.66 | 3.02 | 4.3 |
| BUCM2a | 0.56 | 2.43 | 4.3 |
| CDM36v | 0.71 | 3.05 | 4.3 |

* Expressed as μg. phosphorus per mg. of dry cells.
Virulent strains are designated as v; avirulent, as a.

varied from a low of 0.39 μg. per mg. of dry cells (strain Ba1 (14)a) to a high of 1.01 μg. (strain Ba1(27)v). RNA phosphorus content varied from 2.32 μg. P per mg. of dry cells (strain Ba1 (9)a) to 4.45 μg. (strains Ba1(19)v) and Ba1(23)v). The RNA to DNA ratio ranged from 2.6 (strain Ba1(27)v) to 9.0 (Ba1 (10)a). The average values for the 33 strains were: DNA, 0.47 μg. P per mg. of dry cells; RNA, 3.43 μg. per mg. of dry cells; and the RNA to DNA ratio, 4.3. To evaluate further variation occurring among the different strains, the standard error of the mean was calculated. For DNA this value was 0.03 μg. P. In the case of RNA the figure was 0.14 μg. P.

TABLE 2

*Nucleic acid phosphorus content of Bacillus cereus strains*

| Strain number | DNA* | RNA* | RNA to DNA |
|---|---|---|---|
| Ba2(37) | 0.41 | 3.10 | 7.6 |
| Ba2(59) | 0.60 | 3.10 | 5.2 |
| Ba2(60) | 0.48 | 3.64 | 7.6 |
| Ba2(61) | 0.45 | 2.49 | 5.5 |
| Ba2(62) | 0.34 | 2.98 | 8.8 |
| Ba2(63) | 0.36 | 2.52 | 7.0 |
| Ba2(65) | 0.46 | 3.40 | 7.8 |
| Ba2(66) | 0.46 | 3.72 | 8.1 |
| Ba2(68) | 0.38 | 5.00 | 13.2 |
| U of H | 0.57 | 2.74 | 4.8 |

* Expressed as μg. phosphorus per mg. of dry cells.

Table 2 presents the values obtained from nucleic acid phosphorus analyses of 10 strains of *B. cereus*. Again there is variation in nucleic acid content among the strains. The difference is particularly marked in the RNA and ratio values. Average values calculated for the various determinations were: DNA. 0.45 μg. P; RNA, 3.27 μg. P; and the ratio, 7.6. The standard errors of the mean were 0.03 μg. P for DNA and 0.23 μg. P. for RNA.

To determine whether the variations observed between different strains might not also be reflected in a variation between the values obtained from multiple analyses of one strain, and therefore suggest a defect in our methodology rather than a true variation among strains, 10 separate sets of determinations simi-

lar to those reported in Tables 1 and 2 were carried out on a single strain of *B. anthracis*. These results are shown in Table 3. Al-

TABLE 3

*Nucleic acid phosphorus content of Bacillus anthracis, strain Ba1(18)a*

| Determination | DNA* | RNA* | RNA to DNA |
|---|---|---|---|
| 1 | 0.50 | 2.59 | 5.2 |
| 2 | 0.50 | 2.87 | 5.7 |
| 3 | 0.53 | 2.94 | 5.5 |
| 4 | 0.51 | 2.94 | 5.8 |
| 5 | 0.39 | 2.94 | 7.5 |
| 6 | 0.55 | 2.87 | 5.2 |
| 7 | 0.47 | 2.01 | 4.3 |
| 8 | 0.50 | 2.93 | 5.9 |
| 9 | 0.49 | 3.01 | 6.1 |
| 10 | 0.38 | 2.21 | 5.8 |
| Avg. | 0.48 | 2.73 | 5.7 |

* Expressed as μg. phosphorus per mg. of dry cells.

though variation does exist, it is not as great as that exhibited in Tables 1 and 2. Calculation of the standard error of the mean gave the following values for DNA, 0.02 μg. P, and for RNA, 0.11 μg. P. Since these figures are less than those obtained by similar calculations from Tables 1 and 2, it would suggest that more variation exists between the analyses of different strains than from multiple determinations carried out on an individual strain.

## *Discussion*

The results presented emphasize the statement of Winder and Denneny (1956) that the most striking feature of nucleic acid analyses is the variability of the results. Tables 1 and 2 establish that the nucleic acid content of various strains of the same species of microorganisms varies within wide limits. Table 3 demonstrates that the variation is indicative of strain differences, and is not caused by an inconsistency in the method. Calculation of the standard error of the mean reveals that there is less variation from the mean RNA and DNA content of one strain than there is in several strains of the same species.

It is of interest to note that there is greater variation in the RNA content than in the DNA. Since DNA is considered to be

associated with the nucleus and RNA with the cytoplasm of cells this fact suggests a greater uniformity of nuclear material among different strains of the same species than of cytoplasmic constituents. The constancy of the DNA content of animal nuclei from the same species has been reported (Vendrely, 1955), and the results of the present investigation indicate that the DNA content of the same species of microorganism also might be a rather constant characteristic.

Stokes and Gunness (1946) have reported that the amino acid composition of a particular strain of microorganism is stable when the organism is grown under fixed conditions, but that the composition varied from strain to strain. The data for the nucleic acid and the amino acid composition suggest that bacteria have a biochemical individuality among members of the same species similar to the variability that has been demonstrated by Williams (1956) for higher forms of life.

This investigation emphasizes the importance of specifying the strain designation of microorganisms employed for quantitative analytical analyses. In this connection, it is interesting to point out that the values obtained by Fitz-James (1955) for the nucleic acid content of *B. cereus* vegetative cells fall within the range of values listed in Table 2. However, direct comparison of the studies would be misleading, for Fitz-James employed 3–4 hr. old cultures while the present investigation utilized 18 hr. old cultures. As pointed out by Stokes and Gunness (1946), alterations of the growth conditions will cause a variation in the composition of the bacterial cell.

A particularly interesting finding (Table 1) was the marked difference between the DNA content of avirulent and virulent strains of *B. anthracis*. Those organisms whose strain designation ends in "a" were stated to be avirulent, while those ending in "v" were stated to be virulent for mice. As can be seen in Table 1, the virulent strains almost uniformly contained more DNA than did avirulent strains. The apparent relationship between virulence and DNA content is clearly shown in Figure 1. Only one virulent strain lay within the range of the DNA content of avirulent organisms. The average DNA phosphorus content of avirulent organisms was 0.54 $\mu$g. per mg. of dry weight of cells while the average content of virulent organisms was 0.80 $\mu$g. Statistical analysis by the variable-squared method of the data presented in

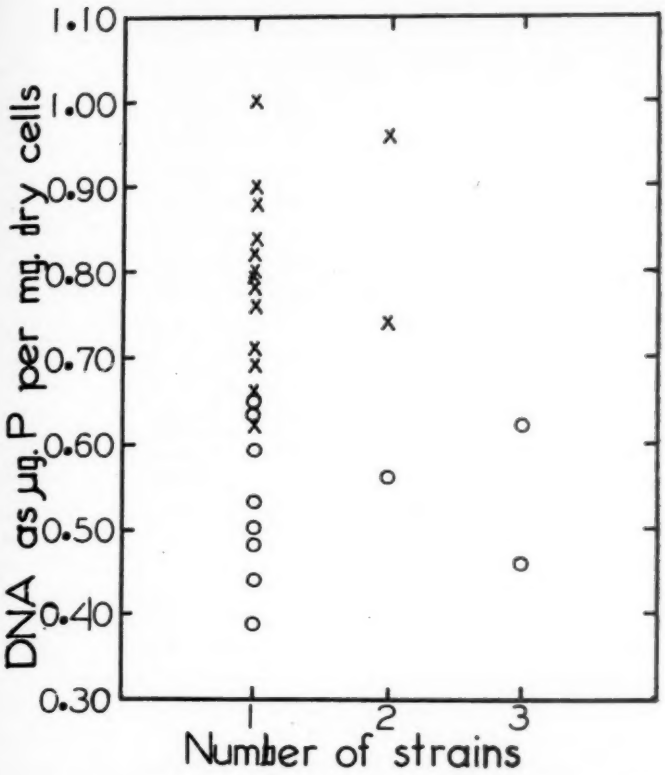


FIG. 1. Diagram showing the relationship between DNA phosphorus content and virulence or avirulence of *B. anthracis* strains. X indicates virulent organism; 0, avirulent organism.

Figure 1 gave a value of $t = 8.6$ which for 31 degrees of freedom yields a value of $P<0.001$. Thus the observed difference in DNA content between avirulent and virulent strains appears to be highly significant.

Overend *et al.* (1951) have reported a difference in the DNA content of avirulent and virulent strains of *Hemophilus pertusis*. If the Schmidt and Thannhauser (1945) method of nucleic acid extraction was employed, they found that avirulent organisms contained less DNA than virulent organisms. However, if the

nucleic acid fraction of the cells was the first extracted by a milder procedure, and the DNA content determined, the results were reversed, avirulent organisms containing more DNA than virulent ones. Explanation of this discrepancy is not apparent.

The results of Overend *et al.* (1951) and those reported in the present paper strongly suggest that a difference may exist in the nucleic acid content of avirulent and virulent strains of the same species of microorganisms. The basis of this difference, and the importance of it with respect to virulence, must await further investigation. It would be of interest to determine whether the difference in DNA content of the organisms also is reflected in a difference in their nucleic acid metabolism.

## *Summary*

The deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) phosphorus content of the vegetative cells of 33 strains of *Bacillus anthracis* and 10 strains of *Bacillus cereus* were determined using the method of Schmidt and Thannhauser. All strains were grown under identical conditions before analysis. Wide variation was found in the content of both species. The DNA content of *B. anthracis* varied from 0.39 μg. P per mg. of dry cells to 1.01 μg. P, with an average value for the 33 determinations of 0.67 μg. P. Avirulent strains contained less DNA than virulent ones. In the case of *B. cereus*, the average DNA content was 0.45 μg. P per mg. of dry cells. The RNA content of *B. anthracis* varied from 2.32 μg. P per mg. of dry cells to 4.45 μg. P. The average content was 3.43 μg. P. The 10 *B. cereus* strains contained an average RNA content of 3.27 μg. P per mg. of dry cells. As would be expected from the DNA and RNA data, the RNA to DNA ratio of individual strains also differed. Multiple determinations of the DNA and RNA content of one strain of *B. anthracis* indicated that there was less variation in this case than when several different strains were examined.

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# MEASUREMENT OF THE PROTECTIVE EFFECT OF ANTIBODY IN BURNED AND UNBURNED PATIENTS' SERA FOR *PSEUDOMONAS AERUGINOSA* INFECTED MICE*

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The failure of presently available antimicrobials to prevent pseudomonad bacteremia consequent on severe thermal injury has focused attention on serum antibody for combating a condition frequently accompanying burn death.(11) Evidence that antibody may play such a role is not conclusive. Fox and Lowbury in 1953 in a paper dealing with bacterial agglutinin to *Pseudomonas* in burned patients reported that poor graft takes and high mortality were associated with greatly elevated serologic titers to this organism.(2) Subsequently, however, these investigators demonstrated specific protective activity of antibody by showing that immunized rabbits developed the smallest lesions when the immunizing strain was used for challenge.(3)

Rosenthal, *et al.*(10) felt a gamma globulin factor prevented death in mice given plasma following *Pseudomonas* challenge. Millican and Rust (8) showed crude rabbit antisera prepared to two septicemic burn strains to be 250–800 times more effective than gamma globulin in protecting mice against fatal infection.

Recently Berk, *et al.* (1) reported endogenous and substrate oxidase activity of *Pseudomonas* to be tremendously stimulated in the presence of animal serum. Equally stimulating, however, was hyperimmune serum made to the organism used for the respirometry studies.

The object of this study was to determine if antibody in the sera of burned and unburned individuals was indeed protective for mice against induced fatal *Pseudomonas* infection. Two types of antibody were studied: 1) a naturally occurring hemagglutin to *Pseudomonas* polysaccharide (PC-9) demonstrated to be present in all healthy adult sera, and 2) immune agglutinin which is present presumably only in the infected person.

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### *Materials and Methods*

*Organisms. Pseudomonas aeruginosa* (DLR strain) phage type 16,44,68,73, 91,109,* a septicopyemic strain isolated from a patient who died following a 70 per cent burn was the challenge organism given mice throughout the testing. A second *Pseudomonas aeruginosa* strain (CP), phage type 16,21,24, 68,73,91, a septicemic strain, was used to prepare 0 antigen for serologic manipulations. PC-9,† a *Pseudomonas* polysaccharide fraction, was the erythrocyte sensitizing antigen employed for assessing native hemagglutinin. All lysotypes of *Ps. aeruginosa* isolated from burned humans at this laboratory possess a slime layer from which PC-9 is recovered. It is, therefore, felt to be an antigen common to all burn pseudomonads.

*LD-50.* Employing the Reed-Muench method (8) the $LD_{50}$ for the Webster-Fairfield mouse (17–22 Gm) was found to be $4.2 \times 10^3$ organisms (17 hour Trypticase broth culture) added in 0.5 ml volume to an equal volume of 5 per cent hog gastric mucin. Ten to 20 times the $LD_{50}$ number of organisms were used to achieve an $LD_{100}$ in these mice.

*Sera.* Single serum samples were obtained from 10 unburned humans while sequential sera (admission, 2 patients), (1–8 day postburn, 10 patients), (8–15 day postburn, 10 patients), and (terminal, 5 patients) were drawn from a total of 10 burned patients. Hyperimmune antibody was obtained by immunizing an experimentally burned white rabbit to the 0 antigen of the DLR strain. All sera were inactivated for complement activity prior to use.

*PD-50.* The protective dose of the sera for 50 per cent of the test animals ($PD_{50}$) was determined utilizing the Miller-Tainter method. (7)

*Procedure.* Thirty minutes after the intraperitoneal injection of the challenge organism into mice, these animals received via the same route 0.25 ml of the undiluted sera or varying saline dilutions of the sera. Previous pilot studies had shown the $PD_{50}$ to be of low dilution so that in addition to undiluted sera, 1:10, 1:50, and 1:100 dilutions were chosen for the testing. Animal mortality was assessed 24 hours following inoculation. Statistical evaluation of the data was not performed because of the paucity of sera from some of the burned patients.

## *Results*

Table 1 shows results obtained in the mice injected with a lethal dose of *Pseudomonas aeruginosa* and inoculated with sera obtained from 10 non-burned individuals whose geometric mean hemagglutinin titer was 1:24. Although undiluted sera from

* The numbers listed are provisional designations for 21 phages recovered from sewage and two phages recovered from previously lysogenic *Pseudomonas aeruginosa* strains employed for phage typing clinical pseudomonad isolates.

† The PC-9 polysaccharide was obtained courtesy Dr. L. C. Ginger, Baxter Laboratories, Morton Grove, Illinois.

TABLE 1

*Per cent survivors and geometric mean hemagglutinin, bacterial agglutinin titers for grouped non-burned and burned human sera*

| Sera | Number of individuals | Number of sera | Number of animals | Geometric mean titers | | Number and percent survivors—24 hours | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | HA | BA | Undiluted sera | 1:10 dilution | 1:50 dilution | 1:100 dilution |
| Non-burn | 10 | 10 | 258 | 1:24 | 0 | 36/66<br>54.54 | 11/60<br>18.33 | 8/66<br>12.12 | 5/66<br>7.57 |
| Burn admission | 2 | 2 | 36 | 1:22 | 0 | .....<br>q.n.s.* | 1/12<br>8.3 | 2/12<br>16.6 | 0/12<br>0 |
| 1st–8th day postburn | 10 | 10 | 240 | 1:9 | 0 | 35/60<br>58.33 | 2/60<br>3.33 | 4/60<br>6.67 | 4/60<br>6.67 |
| 8th–15th day postburn | 10 | 10 | 240 | 1:14 | 0 | 32/60<br>53.33 | 2/60<br>3.33 | 4/60<br>6.67 | 6/60<br>10.00 |
| Expired (CP serum included) | 5 | 5 | 120 | 1:13 | 1:103 | 11/30<br>36.67 | 9/30<br>30.00 | 8/30<br>26.67 | 7/30<br>23.33 |
| Expired (CP serum excluded) | 4 | 4 | 96 | 1:8 | 0 | 5/24<br>20.83 | 4/24<br>16.67 | 3/24<br>12.50 | 2/24<br>8.33 |

* Quantity insufficient for undiluted use.
HA hemagglutinin.
BA bacterial agglutinin.
CP single expired serum from a patient with a protracted septicemic course.

these individuals were able to save slightly more than 50 per cent of the animals, low log dilutions of the sera resulted in a sharp

decline in protective function ($10^{-1}$, 18 per cent survivors; $10^{-2}$, 7 per cent survivors).

Twenty-two sera obtained from burned patients ranging from day of admission to the fifteenth postburn day having a hemagglutinin titer from 1:9 to 1:22 demonstrated equally poor protection in log dilution for pseudomonad infected mice. The undiluted sera of the 1 to 8 day segment of this group were not significantly different, but log dilutions of this sera proved considerably less effective than the non-burn sera. Burn sera from the 8 to 15 postburn day period generally possessed the same protective capability as nonburn sera. It was possible to secure only two admission sera, and since this was of scant quantity, only the diluted sera were used. A $10^{-2}$ dilution of this sera failed to protect any of the mice inoculated.

Five sera obtained, immediately following death, from burned patients provided the best overall protection for the mice. Although the undiluted sera from this group were less protective than the nonburn sera, a $10^{-2}$ dilution still protected 23 per cent of the animals. It is evident, however, from the last line of figures in Table 1 that total expired sera minus a single serum (CP) possesses only the same protection as nonburn sera for mice at this dilution (expired 8.33 per cent; nonburn, 7.57 per cent).

TABLE 2

*Number of animals protected by three sera samples obtained from patient CP*

| Serum 2nd day postburn | | Serum 10th day postburn | | Serum 42nd day postburn | | | |
|---|---|---|---|---|---|---|---|
| HA(PC-9) | 1:4 | HA(PC-9) | 1:16 | HA(PC-9) | 1:128 | | |
| BA | 0 | BA | 0 | BA | 1:512 | | |
| HHA | 0 | HHA | 0 | HHA | 1:1024 | | |
| AT | 0 | AT | 0 | AT | 1:4 | | |
| $PD_{50}$ | <1:10 | $PD_{50}$ | <1:10 | $PD_{50}$ | 1:236 | | |
| Serum dilution | Number of survivors | Serum dilution | Number of survivors | Serum dilution | Number of survivors | Serum dilution | Number of survivors |
| Undiluted | 6/10 | Undiluted | 9/10 | Undiluted | 10/10 | 1:300 | 3/10 |
| 1:10 | 2/10 | 1:10 | 1/10 | 1:10 | 10/10 | 1:400 | 1/10 |
| 1:50 | 1/10 | 1:50 | 2/10 | 1:50 | 10/10 | 1:500 | 4/10 |
| 1:100 | 3/10 | 1:100 | 2/10 | 1:100 | 8/10 | 1:700 | 0/10 |
| | | | | 1:200 | 6/10 | 1:1600 | 0/10 |

BA Bacterial agglutinin titer

HA Hemagglutinin titer obtained with PC-9 sensitized rbcs.

$PD_{50}$ Titer of sera which was capable of protecting 50 per cent of the animals inoculated.

HHA Homotypic hemagglutinin titer assessed with rbcs sensitized with lipopolysaccharide obtained from patient's organism.

AT Titer obtained with PC-9 sensitized rbcs after serum was absorbed with patient's organism.

Table 2 shows results obtained with sequential sera obtained from the clinical course of CP in more detail. The protective dose of these sera, unmeasurable as late as postburn day 10, was found on the forty-second postburn day to have a $PD_{50}$ of 1:236. The hemagglutinin to PC-9 polysaccharide rose from a titer of 1:4 on the second day after admission to 1:128. Similarly, the bacterial agglutinin for the DLR strain showed an increase from zero to 1:512; the homotypic hemagglutinin was even higher. This hemagglutinin to an antigen made from the patient's organism gave a titer of 1:1024. Absorption of forty-second postburn day serum with the patient's organism removed all agglutinin except a small fraction reactable with PC-9 at the original titer of 1:4.

A comparison of the CP sera antibody with hyperimmune antibody from an experimentally burned rabbit, which it resembles, was of interest. Table 3 shows a smaller increase for the

TABLE 3

*Number of animals protected by normal and hyperimmune rabbit sera*

| Normal rabbit serum | | | Hyperimmune rabbit serum | | |
|---|---|---|---|---|---|
| HA(PC-9) | 0 | | HA(PC-9) | 1:4 | |
| BA(DLR) | 0 | | BA(DLR) | 1:256 | |
| HHA(DLR) | 0 | | HHA(DLR) | 1:512 | |
| | | | AT | 0 | |
| $PD_{50}$ | <1:10 | | $PD_{50}$ | >1:1000 | |
| Serum dilution | Number of survivors | Serum dilution | Number of survivors | Serum dilution | Number of survivors |
| Undiluted | 3/6 | Undiluted | 6/6 | 1:400 | 6/6 |
| 1:10 | 2/6 | 1:10 | 6/6 | 1:800 | 6/6 |
| 1:50 | 3/6 | 1:100 | 6/6 | 1:1000 | 6/6 |
| 1:100 | 1/6 | 1:200 | 6/6 | | |

HA Hemagglutinin titer obtained with PC-9 sensitized rbcs.
BA Bacterial agglutinin titer (DLR strain).
HHA Homotypic hemagglutinin titer assessed with rbcs sensitized with lipopolysaccharide obtained from DLR strain.
AT Titer obtained with PC-9 sensitized rbcs after serum absorbed with DLR organism.

PC-9 hemagglutinin in the rabbit serum. Absent in pre-immunization serum, it rose only to 1:4. Bacterial hemagglutinin and homotypic hemagglutinin increased concurrently rising to 1:256 and 1:512 respectively, by the end of the immunization period. The protective quality of this serum was found to be in excess of 1:1000. Absorption of the serum with the immunizing organism left no antibody reactable with PC-9 antigen.

*Discussion*

It would seem from the results of the limited study described here that the hemagglutinin to PC-9, reactable as native antibody to *Pseudomonas* in normal and burned individuals, has protective function only when undiluted sera is used for mouse protection. Dilution of such sera causes a decided drop in protective quality. Inability to dilute such sera without loss of protection would preclude its use in passive immunization. Clinical verification of this is the fact that almost all patients hospitalized in this unit have measurable PC-9 hemagglutinin, but none have survived Pseudomonas septicemia (11) and/or endotoxemia leading in some cases to generalized Shwartzman sensitization. (6)

While protective efficacy of the native hemagglutinin is suspect, it is difficult to evaluate the protective role of hyperimmune bacterial agglutinin or hemagglutinin from sera of burn cases. Only in one patient (CP) was it possible to collect sequential sera, but as previously stated, this is because a burned patient's hospitalization is very rarely protracted following the onset of *Pseudomonas* septicemia.

In the case of CP sera certain data are of interest. Shortly after admission this man's serum contained only a small amount of antibody to the PC-9 antigen. Mouse protection was not present in this serum. By the tenth postburn day no change was evident except that the PC-9 hemagglutinin had increased four-fold. It was shortly after this time that patient CP experienced a remarkable series of 15 positive blood cultures for *Pseudomonas*. The forty-second postburn day serum, taken just prior to death, showed an apparent correlation between good protective titer antibody ($PD_{50}$ 1:236) and the two hemagglutinins and the bacterial agglutinin. It is of interest to note, however, that in spite of the fact that both the homotypic hemagglutinating titer and the bacterial agglutinating titer rose sharply during the 42 day period of observation, the rise in anti-PC-9 titer was only moderate, even though the PC-9 antigen is known to be in the slime layer of all the lysotypes tested. Absorption of the serum with the patient's organism removed all but a small fraction of the hemagglutinin directed toward the PC-9 antigen. No workable amount of absorbed serum remained at this point which would have permitted mouse testing to determine protective quality of this fraction.

That protection perhaps resides in hyperimmune antibody is further suggested by the rabbit antisera data. Prior to immunization no antiobdy titer to any of the three antigens was demonstrable, and the protection for mice was nil. Following conventional immunization which produced a bacterial agglutinin titer to the immunizing strain of 1:256 and a homotypic hemagglutinin titer of 1:512, the $PD_{50}$ value rose to greater than 1:1000. At the same time there occurred only a small rise in hemagglutinin to PC-9. This was removed by absorption of the rabbit antiserum with the immunizing strain. Again the heterologous reaction between PC-9 and the homotypic antigen perhaps explains the increase in the hemagglutinin to PC-9. It would seem that the native hemagglutinin cannot be identified with protection in the case of CP; neither is it stimulated anamnestically by related pseudomonad antigens.

It is difficult to assess the significance of elevated anti-pseudomonas titers in the burn patient. Fox and Lowbury, while noting an association of greatly elevated titers with greater graft failure and higher mortality, nonetheless point out that this association may be nothing more than a reflection of the severity and extension of the burn area. (2) In animal experiments by contrast, (3) they found that intradermal lesions in rabbits were always consistently smaller when the challenging organism was the immunizing strain.

In the case of patient CP, death occurred following septicemia of several weeks duration and at the time when the immune bacterial agglutinin, the homotypic hemagglutinin, and the mouse protective antibody titer were of high order. His death may be ascribed to one or several stress factors, ranging from bacterial endotoxemia to the so-called "burn-toxin". (6) It is believed by the authors, however, that production of immune antibody by the host permitted the phenomenon of 15 successive positive blood cultures in this patient prior to death. And it is, of course, possible that the $PD_{50}$ value of 1:236 may still be far below what was ultimately needed to tip the balance in the patient's favor.

Previously none of our burned patients with two successive daily blood cultures positive for *Pseudomonas aeruginosa* have survived beyond 72 hours. Since it is highly unlikely in this short period that immune antibody would develop of sufficient titer to

be protective, no fair clinical or *in vitro* evaluation of this serum fraction is made. Although only one patient is involved, these results are in agreement with the work of Millican (8) who protected almost all of his mice from fatal *Pseudomonas* infection with crude antisera made to two *Pseudomonas* isolates. This group currently is using hyperimmune rabbit sera for burn cases in children in Peru. Preliminary reports are extremely favorable.

## *Summary*

The protective efficacy (PD-50) of serum antibody measured as native hemagglutinin from burned and unburned individuals for mice inoculated with *Pseudomonas aeruginosa* was found to be small.

In one burned patient where it was possible to secure sequential sera to study immune antibody produced to the infecting organism a high degree of protection for mice (PD-50 1:236) was noted.

That immune bacterial agglutinin and hemagglutinin are intimately related to protection for mice was substantiated by absorption procedures of this human sera and hyperimmune serum made in a rabbit.

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# THIOESTER ANALOGS AS NON-COMPETITIVE METABOLIC ANTAGONISTS[a]

KYOZO HAYASHI,[b] CHARLES G. SKINNER AND WILLIAM SHIVE

Analogs of glutamine containing reactive groups such as O-diazoacetyl-L-serine (azaserine) (6), 6-diazo-5-oxo-L-norleucine (DON) (6), and S-carbamylcysteine (8) exert inhibitory biological effects which are not competitively reversed by glutamine. Glutamine only competitively delays the inactivation of certain enzymes interacting with these analogs. Since an essential metabolite or nutrient present in the host may frequently prevent its competitive antagonist from exerting a chemotherapeutic effect, the introduction of reactive groups into potential metabolic antagonists was undertaken. In this investigation, thioesters of *p*-aminobenzoic acid, *p*-aminosalicylic acid and phenylalanine were prepared and found to inhibit growth of certain microorganisms, and only partial reversal of the inhibitions could be achieved even with natural extracts.

## *Experimental Procedures*[*]

### *Biological Assays*

For the assays using *Leuconostoc dextranicum* 8086, a previously described amino acid medium (7) was modified by adding 0.2 γ/ml. of calcium pantothenate, 0.02 γ/ml. of pantethine, and by increasing the Salts A concentration four-fold. For *Streptococcus lactis* 8039 and *Lactobacillus arabinosus* 17–5, the same original medium was employed except that pantothenic acid was added at a concentration of 0.2 γ/ml. For *Escherichia coli* 9723, a previously described salts-glucose medium (2) was used under assay conditions which have been reported (4). *E. coli* was incubated at 37° for about 12 hours, and the other three organisms were incubated at 30° for about 18 hours.

The thioester derivatives were freshly prepared in ethanol solution at 10 mg./ml. just prior to the assay, and were immediately diluted in sterile water to give a solution of 100 γ/ml. from which additions to the assay tubes were made. At higher aqueous concentration levels, the derivatives precipitated

[a] Contribution from The Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas, Austin. Received for publication January, 1961.

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[*] All melting points are uncorrected. The authors are indebted to Dr. J. M. Ravel and Mrs. J. Humphries for assistance with the microbiological assays, and to J. D. Glass and C. Hedgcoth for the elemental analyses.

from the assay medium. The amount of growth was determined turbidimetrically in terms of galvanometer readings adjusted such that distilled water reads 0 and an opaque objects 100.

*Organic Syntheses*

*DL-Phenylalanine Thioisopropyl Ester Hydrochloride.*—Using a previously reported technique, (5) 5 g. of DL-phenylalanine suspended in 100 ml. of acetyl chloride were treated with a total of 9.5 g. of phosphorus pentachloride for about 3.5 hours. There were recovered 4.5 g. of the corresponding acid chloride hydrochloride, m.p. 145–147°. A sample of 0.7 g. of phenylalanyl chloride hydrochloride suspended in 10 ml. of benzene was treated with 2.5 g. of isopropylthiole, and the reaction mixture was heated at 70–80° for about 10 hours to effect complete solution. The resulting solution was then concentrated *in vacuo*, and the residue was washed with ether. The resulting product was crystallized from ethanol-ether to yield 0.65 g. of product, m.p. 181–182° dec.

*Anal.* Calcd. for $C_{12}H_{17}NOS{\cdot}HCl$: C, 55.50; H, 6.94; N, 5.39. Found: C, 55.68; H, 6.87; N, 5.25.

*DL-Phenylalanine Thiobenzyl Ester Hydrochloride.*—A mixture of 1.5 g. of phenylalanyl chloride hydrochloride and 5 ml. of benzylthiol in about 5 ml. of benzene was heated on a steam cone for about 2 hours. The reaction mixture was then reduced *in vacuo* to yield a residue which crystallized upon treatment with ether. The resulting solid was recrystallized from ethanol-ether to yield 1.8 g. of product, m.p. 117–118° dec.

*Anal.* Calcd. for $C_{16}H_{17}NOS{\cdot}HCl$: N, 4.55. Found: N, 4.78.

*Benzyl p-Nitrothiobenzoate.*—A 3 g. sample of *p*-nitrobenzoic acid was converted to its acid chloride using thionyl chloride, and the resulting acyl halide was taken up in 10 ml. of benzene. To this benzene solution was added 3.5 ml. of benzylthiol and the mixture was heated under reflux for about 12 hours. After cooling, Skellysolve B was added and a crystalline material precipitated from the reaction mixture. The solid was recovered and recrystallized from ethanol-Skellysolve B to yield 2.8 g. of product, m.p. 80–81°.

*Anal.* Calcd. for $C_{14}H_{11}NO_3S$: C, 61.52; H, 4.06; N, 5.13. Found: C, 61.10; H, 4.14; N, 5.40.

*p-Aminobenzoic Acid Thiobenzyl Ester Hydrochloride.*—To a solution of 0.5 g. of *p*-nitrobenzoic acid thiobenzyl ester in 35 ml. of ethanol was added 100 mg. of platinum oxide, and the reaction mixture was then treated with hydrogen gas at 30 lb./sq. in. pressure at room temperature for about 4 hours. After removal of the catalyst, the alcoholic solution was reduced to dryness *in vacuo* to yield an oily product which did not crystallize. This material was subsequently taken up in ether, and anhydrous hydrogen chloride was bubbled through the solution. Upon cooling, a crystalline precipitate formed which was recrystallized from ethanol-ether to yield 0.4 g. of product, m.p. 153–154°.

*Anal.* Calcd. for $C_{14}H_{13}NOS{\cdot}HCl$: C, 60.06; H, 5.05; N, 5.01. Found: C, 59.98, H. 4.70; N, 5.30.

TABLE 1

*p-Nitrosalicylic acid thioesters*

| R | Yield, per cent | M.p., °C. | Empirical formula | Elemental analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Calculated | | | Found | | |
| | | | | C | H | N | C | H | N |
| $C_2H_5$– | 84 | 93–94 | $C_9H_9O_4NS$ | 47.57 | 3.98 | 6.16 | 47.57 | 3.97 | 6.35 |
| $(CH_3)_2CH$– | 56 | 139–41 | $C_{10}H_{11}O_4NS$ | 49.78 | 4.59 | 5.81 | 50.64 | 4.67 | 5.77 |
| $CH_3CH_2CH_2$– | 66 | 82–84 | $C_{10}H_{11}O_4NS$ | 49.78 | 4.59 | 5.81 | 49.61 | 4.74 | 5.70 |
| $C_6H_5$– | 63 | 194–95 | $C_{14}H_9O_4NS$ | 56.78 | 3.29 | 5.09 | 56.52 | 3.44 | 5.09 |
| $C_6H_5CH_2$– | 72 | 89–90 | $C_{14}H_{11}O_4NS$ | 58.13 | 3.38 | 4.82 | 58.63 | 4.10 | 4.93 |

*p-Nitrosalicylic Acid Thioesters (Table 1).*—These compounds were all prepared in essentially the same manner, and a typical preparative procedure is herein described. To a mixture of 5.0 g. of *p*-nitrosalicylic acid in 50 ml. of benzene was added 7.5 g. of oxalyl chloride. After the initial evolution of gas had subsided, the reaction mixture was heated under reflux for about 15 hours. The resulting solution was concentrated *in vacuo* to yield a yellowish oil which was immediately condensed with the appropriate thiol since the acyl halide was quite unstable (1). A solution containing 2 g. of the crude *p*-nitrosalicyl chloride in 20 ml. of benzene was treated with a molecular excess of the desired thiol, and 20 ml. of pyridine were slowly added to the reaction mixture with efficient stirring. In some instances a precipitate began to form at this stage; however, the resulting mixture was then heated under reflux for about 5 hours, and finally reduced to dryness *in vacuo*. The residue was dissolved in acetone, and Skellysolve B was added to induce crystallization of the reaction product. After cooling in the refrigerator for several hours, the crystalline material was recovered and recrystallized from acetone-ether. The individual data on these derivatives are presented in Table 1.

## *Results and Discussion*

The benzylthioester of phenylalanine was prepared directly from phenylalanine through the reaction of the corresponding aminoacyl chloride hydrochloride with benzylthiol. For the *p*-aminobenzoic acid analog, the corresponding *p*-nitrobenzoyl chloride was condensed with benzylthiol, and the resulting thio derivative was catalytically hydrogenated to yield the desired

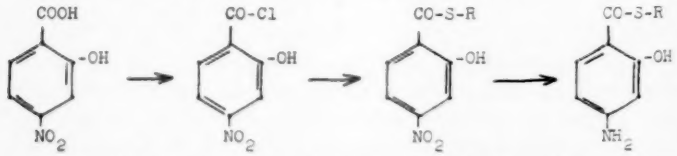
TABLE 2

*p-Aminosalicylic acid thioesters*

| R | Yield, per cent | M.p., °C. | Empirical formula | Elemental analysis | |
|---|---|---|---|---|---|
| | | | | Nitrogen (calcd.) | Nitrogen (found) |
| $C_2H_5$– | 75 | 88–89 | $C_9H_{11}O_2NS$ | 7.11 | 7.09 |
| $CH_3$ \ CH– / $CH_3$ | 80 | 110–113 | $C_{10}H_{13}O_2NSHCl$ | 5.65 | 5.64 |
| $CH_3CH_2CH_2$– | 75 | 108–111 | $C_{10}H_{13}O_2NSHCl$ | 5.65 | 5.46 |
| $C_6H_5$– | 90 | 99–100 | $C_{13}H_{11}O_2NS$ | 5.71 | 5.71 |
| $C_6H_5CH_2$– | 49 | 97–98 | $C_{14}H_{13}O_2NS$ | 5.39 | 5.51 |

*p-Aminosalicylic Acid Thioesters (Table 2).*—The nitro analogs described above were all hydrogenated in essentially the same fashion to yield the corresponding amino derivatives. A solution of 0.25 g. of the appropriate *p*-nitrosalicylic acid thioester in 30 ml. of ethanol was placed in a flask and about 30 mg. of platinum oxide was added. The reaction mixture was shaken in the presence of hydrogen under about 3 atmospheres pressure at room temperature until 3 moles of hydrogen had been absorbed, which usually required about 5 hours. The catalyst was recovered, and the filtrate was concentrated *in vacuo*. In several instances a crystalline residue was obtained directly which was then recrystallized from ether-Skellysolve B; however, in other cases the material failed to crystallize, and it was then taken up in ether and precipitated as a hydrochloride salt using anhydrous hydrogen chloride. A summary of the data on these derivatives is presented in Table 2.

thiobenzyl ester of *p*-aminobenzoic acid. The syntheses of the thioesters of *p*-aminosalicylic acid was carried out as indicated in the accompanying equations starting with *p*-nitrosalicylic acid which was synthesized through a series of previously reported reactions starting from 2-amino-4-nitrotoluene (3).




The biological testing of these *p*-aminosalicylic acid thioesters proved to be difficult owing to their poor solubility in the assay

medium. The maximum level which could be studied was about 20 γ/ml.; and, in addition, these derivatives are destroyed through heating or by standing in solution for a few hours. An attempt is now underway to increase the solubilities of these thioester type derivatives by placing hydrophylic groups on the mercapto side chain, and thus permit biological studies at higher concentration levels.

TABLE 3

*Toxic effects of some thioester analogs*

| Analog | Concentration level necessary for complete inhibition of growth, γ/ml.[a] | | | |
|---|---|---|---|---|
| | *S. lactis* | *L. dextranicum* | *L. arabinosus* | *E. coli* |
| Thiobenzyl ester of DL-phenylalanine | 20 | 6 | >20 | >20 |
| Thiobenzyl ester of p-aminobenzoic acid | 20 | 20 | 20 | >20 |
| Thioesters of *p*-amino-salicylic acid: | | | | |
| Benzyl- | 2 | 6 | 6 | >20 |
| Phenyl- | 2 | 6 | 6 | >20 |
| Ethyl- | 6 | 6 | 6 | >20 |
| Propyl- | 20 | 20 | 6 | >20 |
| Isopropyl- | 6 | 20 | 20 | >20 |
| *p*-Aminosalicylic acid | 2000 | >2000 | >2000 | >2000 |

[a] The solubility of the inactive analogs in the assay media precluded testing them at higher concentration levels.

Thioesters of *p*-aminosalicylic acid (Table 3) were toxic to growth of *S. lactis*, *L. dextranicum* and *L. arabinosus* but not to *E. coli* at levels of from 2 to 20 γ/ml. Preliminary attempts to reverse these growth toxicities using known metabolites and extracts of natural materials indicated that the analogs were only partially reversed by high concentrations of natural extracts and to a lesser extent by certain sulfur containing analogs, e.g., cysteine and sodium thioglycollate. In contrast to the inhibitory properties of its thioester analogs, *p*-aminosalicylic acid was essentially nontoxic in these assay systems even at a concentration level of 2 mg./ml. These initial studies suggest that a thioester linkage substituted in an appropriate metabolite can produce metabolic antagonists which are not appreciably affected by natural reversing agents, and are potentially useful chemotherapeutic agents.

*Summary*

Thioesters of *p*-aminobenzoic acid, phenylalanine and *p*-aminosalicylic acid were synthesized and their toxicities were determined for *Escherichia coli*, *Streptococcus lactis*, *Leuconostoc dextranicum* and *Lactobacillus arabinosus*. In general, the derivatives were inhibitory to growth of the latter three organisms in a manner which suggests that thioester analogs of metabolites may produce noncompetitive antagonists.

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# EFFECT OF MUCIN AND CORTISOL ON RESISTANCE OF MICE TO TWO DIFFERENT STRAINS OF *S. AUREUS* AND THE POSSIBLE IMPORTANCE OF ENDOTOXIN IN THE INFECTIOUS PROCESS[a]

R. D. Higginbotham and Joseph A. Bass[b]

It is well recognized that alterations in the physiologic state of the host may be decisive factors in determining susceptibility to the pathogenic effects of *Staphylococcus aureus*. (5, 7, 10) In this regard, resistance to this pathogen may be markedly altered by such diverse agents as adrenocorticoids (14, 17, 20) and mucin (15, 19, 21). Since these agents affect different and various aspects of host resistance (23, 26) and different strains of *S. aureus* may possess different *in vitro* virulence factors (18), it would seem likely that the overall effects of adrenocorticoids and of mucin on host resistance may vary according to the strain of microorganism involved.

In a study (2) involving a large number of strains of *S. aureus* originally isolated from lesions in humans, we observed that while mice were susceptible to the lethal effects of some strains following injection by the I.V. route and to other strains following injection by the intraperitoneal route, they were not significantly susceptible to any one strain by both routes of challenge. This would suggest that the relative importance of the various tissue factors involved in host resistance may vary according to the strain of *S. aureus* employed. In the present study a strain of *S. aureus* which was virulent for mice following injection by the intravenous route and a strain virulent following injection by the intraperitoneal route were used to determine the respective influences of mucin and of cortisol on resistance of mice to the pathogenic effects of these distinctly different strains of *S. aureus*.

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[b] From the Department of Microbiology, The University of Texas Medical Branch, Galveston. The authors wish to acknowledge the invaluable technical assistance of Mrs. Ursula Hild.

### *Materials and Methods*

Mice: White, female Webster-Fairfield mice weighing 21 to 25 grams were used throughout this study. The animals were obtained from Euer's Farms, Austin, Texas, and after arrival at this laboratory were rested for approximately one week before use. Where employed, bilateral adrenalectomies were performed by the dorsal approach 18 hours prior to bacterial challenge. These animals were fed *ad libitum* and maintained on 0.9% saline as drinking water.

*Bacteria.* The organisms selected for this study consisted of two mouse-virulent strains of *Staphylococcus aureus* which had been originally isolated from human sources: a) 959 strain, originally obtained from blood of a patient at John Sealy Hospital, was virulent for mice by the intravenous route and b) Fritchie strain, originally isolated in the laboratory of Dr. L. P. Gebhardt at the University of Utah Medical School from a granulomatous lesion, was virulent for mice by the intraperitoneal route. The cultural and *in vitro* virulence characteristics of these organisms has been discussed elsewhere (2). The bacteria were prepared for inoculation by growing them for 18 hours on nutrient agar and then transferring them to brain-heart infusion broth for an additional three to four hours of growth. The cells were sedimented from the broth by centrifugation and resuspended with M/15 phosphate buffer (pH 7.4) to the various concentrations required with the aid of a Klett Summerson photocolorimeter (green filter, 540 m$\mu$). Estimates of numbers of viable cells based on turbidity of suspensions were confirmed by plate counting.

*Treatment and challenge.* Hydrocortisone acetate (Hydrocortone, Merck, Sharpe & Dohme) was administered in a dose of 2.5 mg/mouse in a volume of 0.1 ml saline by cubcutaneous injection at 18 hours and again at 2 hours prior to challenge. Mucin (Control H 32–002, Armour Laboratories) was initially prepared as a 50 or 100 mg/ml suspension (pH 7.2) and diluted with buffer to the various concentrations desired for incorporation with the challenge materials. In all instances, the challenge inocula consisted of 0.25 ml buffer containing measured amounts of the respective challenge agents in suspension either with or without an appropriate amount of mucin. Except where noted otherwise, the lethal effects of the various challenges are based on the mortality occurring in the experimental groups during a 10-day post challenge period. Each experimental group consisted of 10 animals and each experiment was repeated at least one time.

## *Experimental Results*

*Effect of route of inoculation.* To quantitatively compare the resistance of mice to the lethal effects of the two strains of *S. aureus* selected for study, groups of mice were challenged with one of a series of graded doses of *S. aureus* of either the 959 or Fritchie strains administered by either the intravenous or intraperitoneal routes. Previous observations indicated that animals almost invariably succumbed to the lethal effects of these infec-

tions within a period of 10 days after challenge. Results from two or more experiments have been accumulated on the basis of the per cent mortality occurring during this observation period and have been presented in Fig. 1. Each point on the graph represents

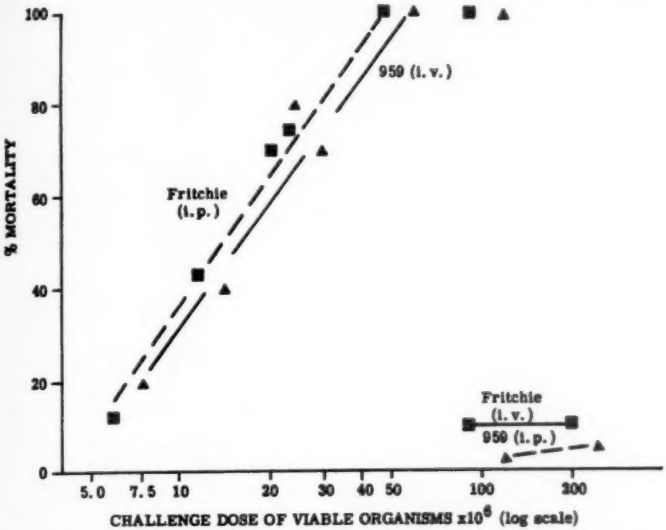


FIG. 1. Comparison of lethal effects of the Fritchie and the 959 strains of *S. aureus* in mice following inoculation by either the intravenous or intraperitoneal routes.

an average of results obtained from 20 to 40 animals. Mice were susceptible to the 959 strain following inoculation of this organism by the intravenous route. This organism killed in a dose response manner in a dose range of 7.5 to 60 million cells per dose with an approximate $LD_{50}$ of 16 million viable cells. In contrast, however, only an occasional death resulted from doses containing as many as 120 to 240 million cells if the challenge was administered by the intraperitoneal route. Thus the local defense mechanisms of the peritoneal cavity were more effective in limiting the lethal effects of the 959 strain following intraperitoneal inoculation than were the systemic defense mechanisms which the bacteria encountered following an intravenous challenge.

Under the same experimental conditions, however, mice were

susceptible to the lethal effects of the Fritchie strain following injection of this organism by the intraperitoneal route in a dosage range of approximately 6 to 50 million viable cells. The $LD_{50}$ was approximately 14 million cells per dose which is not markedly different from the $LD_{50}$ of the 959 strain when inoculated by the intravenous route. However, mice were highly resistant to the lethal effects of the Fritchie strain when it was administered by the intravenous route, even when the challenge was administered in amounts as large as 100 to 200 million cells/dose. As previously reported (14) the systemic defense mechanisms (i.e., the reticuloendothelial system) of the normal mouse are highly effective in resistance to the pathogenic activities of the Fritchie strain, whereas the local defense mechanisms of the peritoneal cavity are relatively ineffective.

Although the $LD_{50}$'s of the Fritchie and 959 strains were found to be quite similar, it was apparent from the results of this experiment, that there must be significant differences in their respective virulence factors since mechanisms of host resistance effective against the 959 strain were relatively ineffective against the Fritchie strain and *vice versa*. This point was further emphasized when the survival times of groups of mice challenged with equivalent doses (approximately $LD_{75}$'s) of either the Fritchie

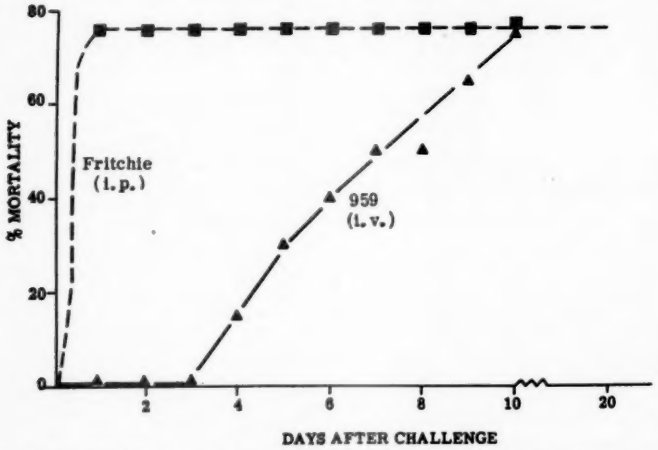


FIG. 2. Survival times of mice following infection with the Fritchie (intraperitoneal route) and the 959 (intravenous route) strains of *S. aureus*.

strain (via the intraperitoneal route) or the 959 strain (via the intravenous route) were compared (Fig. 2). Lethal infections with the Fritchie strain were characterized by the development of an acute state of intoxication (ruffled fur, unsteady gait, etc.) and the animals died within 6 to 24 hours after challenge. Symptoms of infection with the 959 strain were slower to appear, the animals were symptomatic over longer periods of time and the majority of deaths occurred between the 3rd and 9th days after challenge.

*Effect of mucin.* The next experiments compared the effect of mucin treatment on the resistance of mice to the pathogenic effects of intraperitoneally inoculated doses of the Fritchie and 959 strains. In the first experiment, groups of mice were challenged by the intraperitoneal route with one of a series of graded amounts of Fritchie cells suspended in 0.25 ml of a given concentration of mucin. The $LD_{50}$ of the Fritchie infection for each of the mucin doses was determined and, for comparative purposes, the ratio of the $LD_{50}$ of the Fritchie strain in the untreated controls to the $LD_{50}$ in each of the mucin-treated groups was used as a potency index and plotted against the respective dose of mucin employed (Fig. 3). Mice treated with doses of mucin ranging from 5.0 to 12.5 mg of this material were approximately 200 to 1000 times more susceptible to the lethal effects of the Fritchie organism than were the controls. The observed deaths occurred primarily within the first 24 hours after challenge and only an occasional death was ever noted among the animals surviving the initial post-challenge period. Since this acute toxic death was characteristic of the Fritchie infection in untreated mice, it would appear that mucin markedly depressed resistance of the animals to this infectious process but without obviously altering the qualitative aspects of this disease process.

However, when this experiment was attempted with the 959 strain, little evidence of this dose response effect of mucin or of bacteria could be obtained. Although the dose of organism used was approximately 6 times greater than the amount required for an $LD_{50}$ by the intravenous route, deaths occurred only in those animals treated with 7.5 mg or more of mucin and the mortality was not greater than 35 to 60 per cent (Table 1). This is of especial interest since 90 per cent of the deaths occurred within 24 hours after challenge which is in marked contrast to

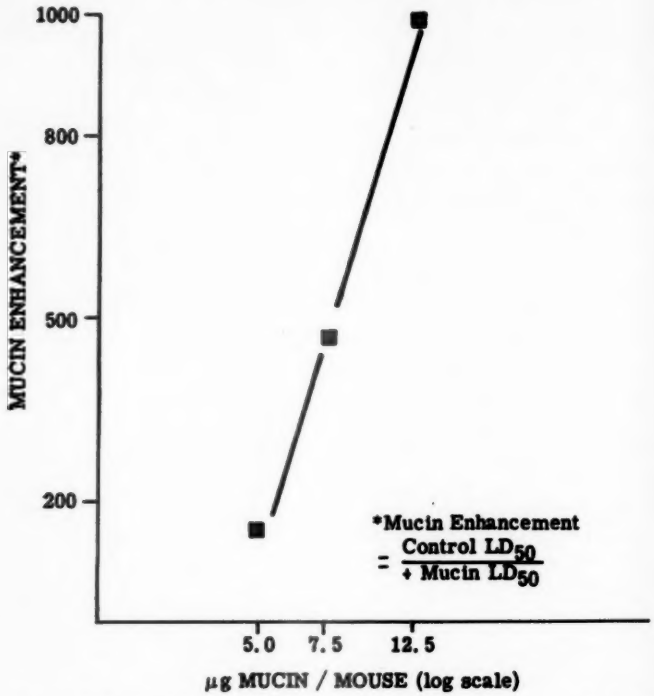


FIG. 3. Enhancing influence of mucin on susceptibility of mice to the lethal effects of the Fritchie strain of *S. aureus*.

the relatively prolonged survival time of mice challenged with the organism by the intravenous route. Thus infection of mucin-treated mice with the 959 strain appeared to be a significantly different process than the disease produced by this same organism when inoculated by the intravenous route. The 959 infection in mucin-treated animals superficially resembled the infection produced in mice by the Fritchie strain.

*Effect of cortisol.* In view of the previous results, it was hypothesized that mucin depressed the activity of some mechanism(s) in the tissues associated with resistance to a virulence factor present in both strains of *S. aureus* but which was very prominent in the Fritchie strain and produced only in limited amounts during infection with the 959 strain. It has been

TABLE 1

*Effect of mucin on susceptibility of mice to the lethal effects of the 959 strain of S.* aureus *inoculated by the intraperitoneal route*

| Mucin (mgm/mouse) | Per cent mortality 24 hours | Per cent mortality 10 days | Per cent total deaths within 24 hours |
|---|---|---|---|
| None | 0 (0/30) | 3.3 (1/30) | ... |
| 2.5 | 0 (0/10) | 20 (2/10) | ... |
| 5.0 | 0 (0/10) | 20 (2/10) | ... |
| 7.5 | 35 (7/20) | 40 (8/20) | 87.5 |
| 10.0 | 60 (6/10) | 60 (6/10) | 100 |
| 12.5 | 50 (20/40) | 62.5 (25/40) | 80 |
| Mucin alone | 0 | 0 | ... |

previously reported that Fritchie possesses a somatic substance whose pharmacologic effects simulate the acute intoxication resulting from infection with this organism (14). In this study, it was observed that adrenalectomized mice were highly susceptible to lethal effects of this material and that pretreatment with cortisol would enhance their resistance. To determine whether a similar substance might be produced during infection with the 959 strain, mice were separated into three groups and treated as noted in Table 2. Animals in each group were then challenged by the intraperitoneal route with inoculums containing either 10 to 20 million Fritchie organisms or 100 million 959 cells. Since this experiment was concerned with evaluating only the acute lethal effect of these organisms, observations were limited to the first 24 hours after challenge (Table 2). Ninety per cent of the adrenalectomized animals failed to survive a challenge with a 50 per cent lethal dose of the Fritchie strain (control) whereas only 10 per cent of the cortisol-treated animals were susceptible to this dose. Since the 959 strain is normally avirulent by the intraperitoneal route, no obvious effects were to be expected in either the control or cortisol-treated groups following injection with the 959 strain, but 30 per cent of the adrenalectomized group were susceptible to the lethal effects of this organism. Thus the

TABLE 2

*Influence of cortisol and of adrenalectomy on resistance of mice to acute lethal intoxication resulting from inoculation of viable* S. aureus *or staphylococcal endotoxin*

| Treatment groups | Per cent mortality | | | |
|---|---|---|---|---|
| | Organisms | | Lipopolysaccharide | |
| | Fritchie ($1–2\times10^7$) | 959 ($2\times10^8$) | No mucin | Mucin (12.5 mg) |
| Control | 50% (10/20) | 0.0 (0/20) | 30% (6/20) | 65% (13/20) |
| Cortisol | 10% (2/20) | 0.0 (0/20) | 0.0 (0/20) | 15% (3/20) |
| Adrenex | 90% (18/20) | 30% (6/20) | .. | .. |

* Data accumulated on basis of mortality occurring during first 24 hours after challenge.

dose of 959 cells, which was approximately 5 to 10 times greater than that of the Fritchie strain, was measurably less toxic. It would therefore appear that if a common factor were present in both strains which was responsible for the acute intoxication observed, this factor was probably produced only in limited amounts during infection with the 959 strain. It was of further interest in this regard to find that a commercially available preparation of staphylococcal endotoxin (Difco, control 116310) produced death in mice within 4 to 24 hours after intraperitoneal injection of doses ranging from 0.2 to 1.0 mg/mouse. Adrenalectomized mice have been found to be more than 1000 times more susceptible to the lethal effects of this drug than are intact controls (unpublished data) and, as noted in the table, pretreatment of intact mice with cortisol enhanced their resistance to the lethal effects of an intraperitoneal challenge dose of endotoxin (0.5 mg) which was lethal to 30 per cent of the controls. On the other hand, mucin effectively decreased resistance of both control and cortisol-treated mice to this dose of endotoxin. However, the steroid-treated animals were more resistant to this mucin-enhanced toxicity of endotoxin than were the non-steroid-treated controls. Since resistance to this endoxic material was depressed by mucin, it would appear probable that the observed decrease in resistance of the mucin-treated mouse to the acute toxic effects of infection with the Fritchie strain and, to a less extent with the 959 strain, could be due to the production of a comparable type of endotoxic substance during the period of infection.

*Effect of cortisol and mucin.* The next experiments were performed to determine whether cortisol would influence the resistance of mucin-treated mice to the lethal effects of infection with the Fritchie or 959 strains as it did to endotoxin. Accordingly, two large groups of mice: a) cortisol-treated and b) untreated controls were separated into a number of smaller groups of 10 animals in each and challenged by the intraperitoneal route with 0.25 ml. of buffer containing: 1) $10^5$ Fritchie cells, 2) $10^5$ Fritchie cells plus 12.5 mg mucin or 3) buffer only. A similar experiment in which $2 \times 10^6$ 959 cells were substituted for the Fritchie organism was run concurrently with the Fritchie experiment. Both experiments were repeated to give a total of 20 animals per individual group. It is clearly evident from Fig. 4

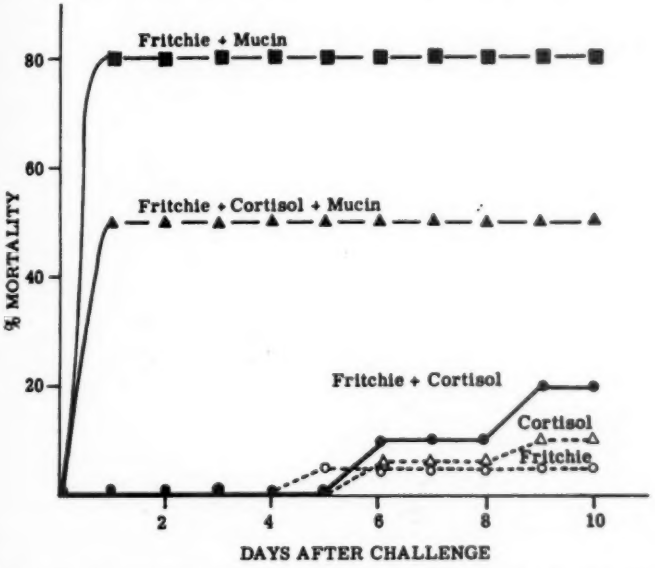


FIG. 4. Influence of mucin and/or cortisol on resistance of mice to the lethal effects of the Fritchie strain of *S. aureus* (intraperitoneal route).

that pretreatment with cortisol markedly enhanced the resistance of mucin-treated mice to the acute pathogenic effects of the Fritchie strain. It is important to note that despite the fact that very large dosages of cortisol were used in this study, only the

protective or antitoxic effect of this hormone was evident in the mucin-treated mice. This beneficial effect apparently did not represent merely an increase in survival time since there was no evidence of additional mortality after the initial 24 hour post-challenge period. On the other hand, in the groups of cortisol-treated mice challenged only with the Fritchie cells (no mucin) there was a slightly greater number of deaths than in the Fritchie-infected controls. These animals died within 4 to 10 days after challenge which is not characteristic of the pathogenic effects of this organism and may possibly represent deleterious effects of cortisol on resistance to infection (23).

It can be seen in Fig. 5 that the acute lethal effects of the 959

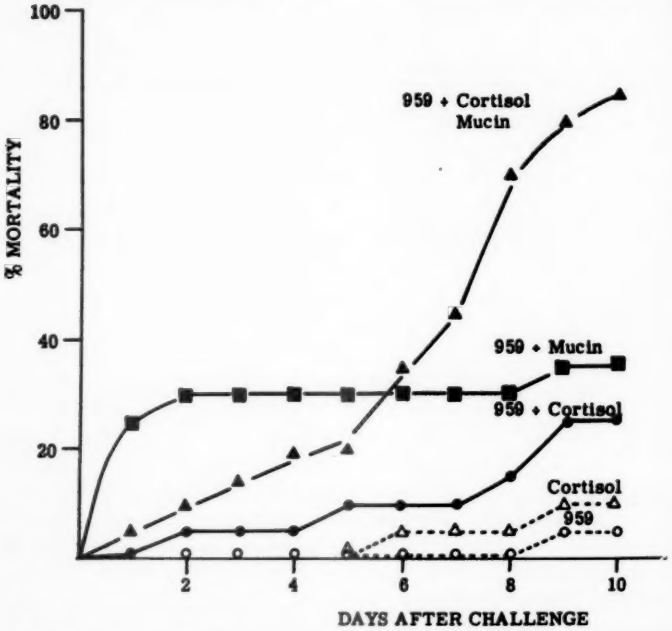


FIG. 5. Influence of mucin and/or cortisol on resistance of mice to lethal effects of the 959 strain of *S. aureus* (intraperitoneal route).

infection in the mucin-treated mice were suppressed by pre-treatment of the animals with cortisol. However, in contrast to the infection with the Fritchie strain, in which deaths are pri-

marily due to this very acute lethal intoxication, the initial protective effect of cortisol was succeeded by a progressive increase in mortality which eventually greatly exceeded the numbers of deaths occurring in the non-cortisol-treated animals receiving the same challenge. This mortality rate was comparable to that resulting from intravenous inoculation of the 959 strain in untreated mice (Fig. 2). The similarity in mortality would suggest that in addition to the anti-toxic effect of cortisol in the mucin-treated mouse, there had also been an inhibition of the ability of local tissue mechanisms to restrict the dissemination of significant amounts of the pathogen into the blood stream through which it could subsequently become established in vital tissues of the host. From the results of these experiments, it would appear that a given amount of cortisol can enhance and/or depress resistance of mice to the lethal effects of staphylococcal infections, dependent on the particular stage of the infectious process being studied and the nature of the particular strain of pathogen employed.

*Discussion.* The disease-producing abilities of different strains of staphylococci are apparently due to a variety of factors whose relative importance varies with the particular strain of organisms involved as well as the physiologic state of the host. Thus the pharmacologic effects of a given pathogenic factor may, with one strain of staphylococci, be obscured by the complexity of the infectious process and, with another strain, be characteristic of the disease. On the other hand, the presence of this factor in the first strain may be revealed by manipulation of the physiologic state of the host to render the animal more susceptible to the pharmacologic effects of this particular factor. Under these conditions the disease produced should resemble that caused by a strain in which this particular factor is dominant.

In the present study, the similarities noted between the effects of staphylococcal endotoxin and of intraperitoneal infections with the Fritchie strain in untreated mice or with the 959 strain in mucin-treated mice as to, 1) time of death (6 to 24 hours), 2) enhanced acute lethal effects in adrenalectomized animals and 3) decreased acute lethal effects in cortisol-pretreated animals leads to the assumption that a similar injurious agent (or agents) was involved in each instance. The enhanced susceptibility of mucin-treated mice to staphylococcal endotoxin as well

as to the infectious agents strengthens this point of view.

It has been clearly indicated that a somatic or endotoxic substance is the active factor responsible for the lethal effects resulting from infection of adrenalectomized mice with the Fritchie strain (14). As noted above, results obtained in the present study suggest that a similar factor may also be present in the 959 strain whose over-all pathogenic effects in mice markedly differ from those of the Fritchie strain. Although endotoxic substances do not appear to have been seriously considered as important factors in the pathogenesis of staphylococcal infections (5, 10), it has been observed that killed preparations of staphylococci are lethal to adrenalectomized rats (25, 13) and that extracts of staphylococci cells may be toxic for guinea pigs (9) and lethal for adrenalectomized mice (14). Moreover, a Boivin-type of extract of staphylococcal cells, as well as viable staphylococci, has been found to enhance the dermal sensitivity of rabbits to epinephrine (1). These effects are similar to those produced by the lipopolysaccharide endotoxins of gram negative bacteria.

In a recent paper on resistance (16), Kass has stated that "In only two situations have adrenocortical hormones been shown to be protective to the host: the replacement of hormone in hypoadrenalism and the protective action against the lethal toxicity of bacterial lipopolysaccharides." It is of interest in this same regard that pretreatment of rabbits (12) or adrenalectomized mice (14) with these hormones has also been reported to enhance the resistance of these animals to the lethal effects of intravenously-injected viable staphylococci. Results from the present and an earlier study (14) indicate that cortisol pretreatment can enhance resistance of adrenalectomized, nonadrenalectomized or mucin-treated mice to the acute lethal intoxication resulting from infection with one or both strains of staphylococci or from an injection of staphylococcal endotoxin. The beneficial effects observed in each instance may be due to the fact that cortisol can serve to satisfy an increased tissue demand for adrenocortical steroids induced by the respective stressful situations. This tissue demand may become progressively more acute as the result of impairment of adrenal function by endotoxic substances (24, 3).

The depressing effect of mucin on resistance to infection is well recognized, but poorly understood. Although interference with

antibacterial mechanisms involving complement (19), properdin (22), or phagocytosis (11) have been suggested, it may very well be that mucin also intereferes with tissue processes which are not directly anti-bacterial in nature, but which are vitally important for the maintenance of the physiologic state of the host during the stress of an infectious process. The decrease in resistance of mucin-treated mice to the acute lethal effects of endotoxin or viable bacteria and the partial inhibition of this mucin effect in cortisol-pretreated animals, as noted in the present study, would suggest that mucin may induce a state of relative adrenal insufficiency in the host. In this regard it should be noted that the change in resistance of the mucin-treated mouse was qualitatively similar to the change following adrenalectomy in that both groups became more susceptible to the acute lethal effects of either the "purified" staphylococcal endotoxin or substances resulting from infectious processes in the tissues. It is of interest that in both instances the Fritchie strain was more potent than was the 959 strain and that the effect of mucin on the pathogenicity of either strain of organism was manifested primarily as an acute lethal intoxication which was very similar to the effects produced by injection of suitable amounts of endotoxin in these animals. Our studies with other strains of *S. aureus* (2) have also suggested that staphylococcal infections in mucin-treated mice are a decidedly different disease from that produced by the respective strains in non-mucin-treated animals and qualitatively resemble the Fritchie or endotoxic type of infection. It may very well be, as has been shown with adrenalectomized (14) and with nutritionally deficient mice (8), that the depressed resistance of mucin-treated mice to the lethal effects of staphylococcal infections may be more closely correlated with a primary inability of these anmals to maintain a physiologic equilibrium than with a marked loss of ability to restrict the proliferation of the pathogen.

The beneficial effects of cortisol on resistance to the lethal effects of an infectious process may oftentimes be obscured by its suppressive effect on various anti-bacterial functions of the tissues such as the inflammatory response and antibody formation which thus permits a greater degree of proliferation and dissemination of the pathogen throughout the tissues of the host. This would appear to be well exemplified by the effect of cortisol

on resistance of mucin-treated mice infected with the 959 strain in which the apparent beneficial effects observed in the early part of the disease were superseded by the progressively increasing mortality among the steroid-treated animals. However this deleterious effect of steroid treatment on antibacterial functions may have been further complicated by a progressively increasing state of adrenal insufficiency in the host brought on by the administration of large doses of steroid and subsequent withdrawal of treatment (4, 6). In a preliminary experiment, we have observed that mice treated with the large doses of cortisol used in the present study became progressively more susceptible to the lethal effects of endotoxin during the 10 day post-treatment observation period. The overall effects of cortisol administration on the outcome of staphylococcal infections in mucin-treated mice are seemingly dependent on the nature of the respective virulence characteristics of these particular strains in infections in untreated mice. That is, the Fritchie strain produces a rapidly lethal intoxication (endotoxic activity) but no obvious complications in animals surviving this early portion of the observation period in contrast to the 959 strain which appears to have the ability to become established in vital tissues ("nidogenic" activity). Since this latter disease process is more chronic in nature, there is an increased opportunity for exploitation of deficiencies in host resistance as they occur. According to this view, cortisol can sufficiently enhance anti-toxic resistance of the host to allow time for the anti-bacterial functions of the tissues to eliminate the Fritchie strain which does not readily establish in the tissues. The resultant physiologic alterations due to hormone treatment are therefore not markedly complicated by progressive injury to vital tissues as they may be during infection with the 959 strain. It may be concluded that resistance of the mouse to lethal infections with staphylococci is dependent on a variety of tissue functions whose relative importance varies with the nature of the particular strain involved as well as the stage of the infectious process observed.

## *Summary*

Resistance to infection with two different mouse-virulent strains of *S. aureus* was studied in mice by comparing the relative influences of various treatments (control, adrenalectomy,

mucin, cortisol and cortisol plus mucin) on their resistance to the lethal effects resulting from intraperitoneal challenge with these pathogens. The organisms used possessed markedly different virulence characteristics in normal mice (i.e., the 959 strain was virulent by the intravenous but not by intraperitoneal route whereas the Fritchie strain was virulent by the intraperitoneal, but not by the intravenous route for challenge). Comparative studies with a commercial preparation of staphylococcal endotoxin suggested that endotoxic substances may play an important role in the production of acute lethal intoxication resulting from infection with these agents. The relative importance of such substances would be dependent on 1) strain of *S. aureus* employed, 2) route of inoculation and 3) physiologic state of the host. In this latter regard, large doses of mucin depressed and large doses of cortisol enhanced resistance to the acute lethal intoxication resulting from infection with either strain or from administration of endotoxin. Moreover, whereas cortisol treatment enhanced the survivorship of mucin-treated animals challenged with the Fritchie strain, this same treatment decreased survivorship of additional mucin-treated mice challenged with the 959 strain. It is considered that the beneficial and/or detrimental effects of cortisol treatment on resistance in experimental *S. aureus* infections in mice is dependent on the *in vivo* virulence characteristics of the particular pathogen employed as well as the tissue requirements for this hormone.

*Addendum:* Subsequent observations (Bact. Proc., 1961) have indicated that either heat-killed or viable cells of the Fritchie strain, and to a lesser extent, of the 959 strain, will induce a hemorrhagic necrosis in the S-180 sarcoma in mice, and that this effect does not markedly differ from that induced by endotoxins from either *S. aureus* or *E. coli*.

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# A STUDY OF "SPECIAL PRESSURES" AND THEIR IMPACT ON THE RELATIONSHIP BETWEEN MOTHERS AND THEIR ASTHMATIC CHILDREN

## A Pilot Study*

HENDRIK LINDT AND ARMOND S. GOLDMAN

### *Introduction*

Until the allergic phenomena were discovered asthma was considered primarily a nervous disease and actually is referred to in older textbooks as "asthma nervosa." With the advent of modern immunology, attention became focussed on the allergic component and the older view of asthma as a nervous disease came to be considered completely obsolete. (2)

More recently, however, in the period of psychosomatic orientation the pendulum swung back again and the emotional etiology of asthma was revived. Being faced with a struggle between obsessive all-inclusiveness and a desire for perspective in summarizing previous asthma research, certain observations can safely be made.

Clinical observations concerning a great variety of potentially precipitating factors in asthma attacks are numerous: these include almost any sudden intensive emotional stimulus such as excitation, jealousy, anxiety and rage. Dr. E. Newhaus, (15) in a recent study (1958) observed that both asthmatic and cardiac children show a similar lack of self-confidence, deep-seated dependency and over-anxiety, and wonders, in conclusion, whether these personality-traits are the result of their chronic illness or due to some other factors.

Dr. Franz Alexander (2) found that the fundamental psychodynamic factor in asthma is a conflict that centers around an excessive unresolved dependency upon the mother. He adds that as a defense against this infantile entanglement "all kinds of personality traits" may develop such as aggressive, ambitious, argumentative persons; but also hypersensitive, aesthetic types

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of individuals, some of whom may turn into compulsive characters, while others may show more of a hysterical nature. He considers any attempt to formulate a characteristic profile as initially attempted by Flanders Dunbar (4a) in relation to psychosomatic patients as futile, since no such profile exists for the asthmatic patient.

Substantial agreement has been reached on one point however, by a majority of observers such as Weiss, (17), English, (17), Alexander (2) and Felix Deutsch, (3) namely that psychic and allergic factors in asthma are more or less complementary in their etiological relationship to each other. Specifically that in some cases asthma attacks may be precipitated by allergic factors alone, in others by emotional factors alone, and still in others the combination of allergic and emotional factors seem necessary to bring about an attack, representing in physiological terms a "summation of stimuli." (2)

French and Alexander (7) report that in a great number of cases studied by them, patients who had undergone psychiatric treatment retained their allergic sensitivity in some form as shown by skin tests, but did lose their asthma, without any desensitization. They feel that this explains the claims of both psychiatrists and allergists regarding the therapeutic efficiency of their respective techniques. French and Alexander then conclude, that in most cases of adult patients it is sufficient to remove one of these two coexisting causative factors to free the patient from his attacks. The untreated factor alone apparently does not suffice in most cases to produce an attack.

These authors finally suggest the possibility that the sensitivity to the separation trauma (regarding the mother) and the sensitivity to allergens frequently appear together in the same person and may be parallel manifestations of the same (constitutional) factor. Separately however, the effect of either type of stimulus is likely to remain below the threshold of sensitivity of the "shock tissue" in this case the wall of the bronchioles, a spasm of which is almost certain to produce the asthmatic attack again and again. (2)

From our observations in the Allergy Clinic, we got the impression that there is a goodly number of asthma cases among children where it is not sufficient to control one of the causative factors to prevent asthma attacks. Specifically it was observed

that even when the known allergic factor appears under control an overwhelming emotional experience may still precipitate attacks.

For all these reasons we concluded quite early that recognition of the emotional factors and a possible control of them, coupled with guidance and therapeutic supportive influence on the mother, presented the most promising avenue of approach towards the interplay between mother and child. This was done in the hope that her attitudes might be modified as long as she was not too advanced in age or too rigid in her ways of dealing with the child and his illness.

Another interesting observation is the tendency to mutual exclusiveness of asthma and psychosis. They may alternate, Ackerman (1) observed, but they usually do not coexist. In those cases where there is both asthma and psychosis, the asthma disappears during the psychosis, and tends to reappear when the psychosis subsides.

In recent times there has been more of a tendency to try to understand the problem in terms of disorganization of specific adaptive mechanisms of personality, i.e. the deviant adaptive operations in pathological formations of defense against real or imagined external dangers and inner anxiety (36).

It is in this area that our study focusses first on a detailed study of the asthmatic child's mother and then on the interplay between mother and child.

The nature of the disturbance in the relationship between the mother and the psychosomatically ill child is regarded by some observers in the literature as being specific to some degree. For instance the mother of the peptic ulcer patient is described as inconsistent and frustrating in her baby-feeding habits; the mother of the colitis patient is careless and sometimes cruel; the mother of the asthmatic patient is smothering and demanding; the mother of the skin patient is given to too much or too little handling. The nature and intensity of the subsequent states of stress influence the susceptibility thus created (3c).

*The Escalona Experiment.* As to the unique quality of interaction between mother and child, an important, although almost forgotten, experiment conducted by Dr. Escalona of the Menninger Clinic (5) should be mentioned. Dr. Escalona reported her findings to the Annual Meeting of the American Ortho-

psychiatric Association at Chicago in 1943. This experiment involved a group of women prisoners at a Pennsylvania State Prison representing an adequate sample who were permitted to care for their infants while serving time.

Her research design provided for an arrangement, according to which a different inmate mother would feed all infants in the prison nursery, including her own, each day. At that time the infants were given a choice of having either orange juice or tomato juice. The investigation, lasting several weeks, showed that almost all infants based their daily choice on the preference which the particular mother, who cared for them on that day, had herself. This meant that an infant who preferred tomato juice yesterday, just like his mother-for-the-day did herself, would express a definite preference for orange juice at any other time if the mother who fed him then preferred orange juice herself.

This experiment is mentioned here mainly to illustrate the almost unbelievable degree of intimacy and ability for nonverbal communication that even infants possess. This in turn explains to some extent the infant's amazing capacity to learn and benefit from his mother's care, as well as the factor of vulnerability as determined by the internal and external pressures under which the family, especially the mother, has to live during the child's formative years.

### *Purpose*

Although the ultimate aim of this project, then, is not limited to its starting point, namely the detailed study of the nonasthmatic mother of the asthmatic child, but rather to an exploration of the interplay of the two, which includes an appraisal of the child, our final goal goes beyond that. We are hoping to clarify what impact the basic personality of the parents, especially the mother, has on their methods of childrearing as to stimulate somatic, non-adaptive responses in their offspring in reaction to emotional stress, for instance responses such as an asthmatic attack. This means that the mother's own background, especially her current life-situation and her past relationships during her formative years, needs to be evaluated in order to understand the internal and external pressures under which she was living, with particular reference to the period and the circumstances prevail-

ing when the asthmatic child came into the family. Last but not least, there was the possibility that the brief interviews carried on with the mothers over a period of time would have a balancing therapeutic influence in the hope of making the attitude of the mother towards her asthmatic child a more realistic and constructive one.

## *Teaching Value*

Beyond this goal of our research project there was a fundamental obligation to make relevant information readily available to medical students and residents on a case by case basis while they were still at a stage in their training that found them receptive and open-minded to the significance of such data.

### *Method*

Our approach was influenced by the idea, which has gained ground in the field of psychosomatic medicine, that families have come to be increasingly considered as one of the main originators and controllers of psychogenic disturbances. Attempts were therefore made to isolate potential predisposing factors and special pressures in the history of the parents and the child, and in particular to clarify the circumstances prevailing at the time of the onset of the illness.

The sample group consisted of 20 children from age five to twelve. We hoped to obtain relevant data by (1) *interviewing* these mothers repeatedly over a period of time at the occasion of their weekly visit to the Allergy Clinic at the Medical Branch; (2) by *observing their behavior* while their children were seen by the medical examiner; and, (3) by *making selective home visits.*

We felt that the stock method of relying on one or two diagnostic interviews would be insufficient to evaluate these complex problems. The aim of these weekly interviews, therefore, was to establish rapport and continuity both with mother and child. It was hoped that this, in turn, would enable the mothers to be less defensive as far as their part in the investigation was concerned. This expectation was largely borne out, since it does take time, and it pays to take the time, to get gradually acquainted with each of the mothers through a series of brief interviews. It was our feeling that these mothers cannot realistically be expected to react any way but evasively and defensively to a single diagnostic interview or a single test session.

Most mothers, we sensed, wanted to be sure that they, as mothers, would not be blamed for the child's asthmatic condition on the basis of faulty or neglectful maternal handling. Only in this way, we believed, could we, within the limitation of our setting, learn what these mothers were like, personality and historywise, and get some information about their family role, their mothering behavior, their goals and standards relating to their children, and at times some data about the influence of cultural patterns on their behavior.

By utilizing such an approach we tried to avoid what might be termed the

"one shot method" used in much of the previous asthma research; which would often focus on a large sample such as a hundred asthmatic patients and possibly their families using a battery of psychological tests and/or a single diagnostic interview with each of the hundred subjects as a basis for their final conclusions.

In order to avoid any feeling of pressure, the interviewer in dealing with each mother would indicate by his attitude that he was not in a hurry, that this or that point might just as well be discussed at some later meeting; all the time making very sure that the mother would not refuse to collaborate in these interviews because the pace was too fast, due to our own determination and vested interest, for her to come to grips with these problems.

In using what by some observers might be considered an unnecessarily tedious approach to the problem, we were influenced by the occasionally heard comment that the dilemma imposed on asthma research by contradictory and insufficiently substantiated data is one which has produced variations of interpretations that are perhaps without many parallels in other fields of medicine. (6)

Of late we have begun to add another step to our investigation which we consider of utmost diagnostic value; namely, *a visit to the homes of selected asthmatic patients* who reside in Galveston county. This will be done in the hope of gaining a better understanding of the patients' situation in the family and the relationship between the parents.

## *Findings and Case Illustrations*

As mentioned above, the goal of our investigation was not limited to an evaluation of the mother's personality but rather to an appraisal of the internal and external pressures under which the members of the family had been living, with special reference to the period and the circumstances prevailing at the time when the asthmatic child came into the family.

Many mothers at first would present a rather calm surface appearance, seemingly carrying on with the care of their children without making any undue fuss. Such a façade often had deceived doctors for long periods of time into focussing all their attention on the sick child, tacitly assuming that the mother was in no particular need of help. That this was not so became repeatedly evident when a mother would break down and cry in response to the interviewer's *first* sign of interest in her as a person who had problems of her own of which the child perhaps was the presenting symptom.

This was one reason why these information-gathering and emotionally supportive interviews with the mothers had to be handled with care. Reference is made here to the fact that asthma

in a young child is usually a wearisome, chronic illness, demanding a great deal of patience and perseverance from the whole family. It was of the utmost importance therefore that the interviewer instead of breaking down a mother's emotional defenses would strengthen and bolster them as much as possible. Otherwise we might have an acutely disturbed mother to look after, with the chances of getting specialized sustained treatment for her not being very great.

Finally the interviewer would not insist that the mother come to the initial interviews alone, leaving the child with the nurse or in the waiting room. However after recognizing that the discussion related in part to their internal and external pressures, all mothers in the sample group without fail would come in alone at the time of subsequent interviews.

Some mothers showed some resistance to the interview because they were basically distrustful or particularly fearful that the blame for the child's asthmatic condition may be fixed on them. Such mothers were given the support and reassurance that they needed. This was done usually by stating, that they as mothers knew more about the child's current life and background than anybody else and that because of their unique position they might be able to help the child in many areas more than an outsider could.

As mentioned above, asthma research in the past suggested the existence of pathological atmospheres during the developmental period of the patient. One of the chief generalizations that was extracted was that mothers of asthmatics were often found to be "smothering and demanding in their attitude" (3c) towards their allergic children. In our project we attempted to find a correlation of specific parental attitudes and the internal and external pressures which brought them about in each case.

Much of the research in the past that featured a diagnostic interview with the mother, used routinely questions such as the following: (6)

> Do you notice any particular circumstances surrounding the asthmatic attack?
> What kind of a child is he?
> What sort of problems does he have?
> Is he an affectionate child?
> Towards whom does he show the most affection?

How does he show affection?
Is he more attached to you, your husband or some other adult?

In our investigation we would find such questions of some value, but they did not lead us to what we wanted to know about the vicissitudes of early family conditioning. For instance we wanted to learn the way in which the mothers were predisposed (on the basis of their own life-history) to remain ineffective in handling their own children and, if possible, to understand what special pressures finally produced the emotional factor that was operative in the child's asthmatic condition.

Among other things we tried to find out, were whether mother and child had been off to a good start and if not, what the reasons for the disturbance in their close relationship had been. As one would expect, these pressures differed in each case. It was our belief that only a greater awareness of the individual family situation would enable us to ease and improve the relationship between parent and child.

*Case Illustration No. 1*

*Patient Inez F. age 7*, is the youngest of six children. There is a difference of sixteen years between her and her oldest sibling. Her mother, now age fifty, and appearing prematurely aged, reported that the patient has always been and still is a physically weak child who vomits when emotionally upset and (in contrast to most adult asthmatics), (2) often cries before and while she experiences an asthmatic attack. The young patient leaves the impression of a pale, intimidated child who by her complexion, posture and general appearance bore out her mother's observation that the patient has a very poor appetite compared to all of her other siblings.

The medical chart confirmed that the patient had been a full term baby and that the pregnancy had been objectively uneventful.

The mother reported that the doctor had advised against nursing her. (This, by the way, was very much in contrast to her own experience, having been nursed by her own mother for twelve months).

At the age of two months, the patient had developed a rash covering face and body. The first asthmatic attack also occurred around that time and prior to the end of the patient's first year of life. In relation to the asthmatic attack, the mother took the patient to the family doctor, but only when the attacks were severe and otherwise the mother waited for three years before making sustained treatment at the Allergy Clinic available to the child. Currently the patient's attacks occur always when she is at home, especially on weekends, and never while the patient is attending school.

The mother was about forty-three years old when the patient was born and winced when she recalled that a nurse at the hospital had referred to the

patient as a "menopause baby". There is little doubt that then, as well as now, the mother's attitude towards this uninvited late-comer, this youngest child of hers, was a negative one leading to a critical disturbance in the phase of the symbiotic union of mother and child.

The patient's three older siblings were born during the period from 1937 to 1941. (The parents had married in 1936). There was then a seven year break during which the mother admitted she had tried to avoid becoming pregnant. Then after seven years she found herself unexpectedly pregnant with her fourth child (1947) and her fifth child (1949) and in 1952 suffered a miscarriage. At that particular time she quoted the doctor attending her as having replied smilingly to her anxious questions that "she was in no danger of further pregnancies."

The mother added that when she realized a few months later that she was nevertheless pregnant again, it came to her as a shock and a surprise.

She spoke with much feeling of how she was kidded and laughed at for having a baby in her middle forties. One woman in the neighborhood told her, "You ought to be ashamed of yourself, having a baby at your age." The mother added with a sigh, "I was younger then, and I was sort of being embarrassed," implying that she was quite suggestible, an attitude which seems to have continued until the present time.

It was our impression that this mother's behavior, while her daughter was examined by the attending physician, was indicative of her basic feelings towards this child: In the examination room she usually stood watching what went on with her arms defensively folded and tapping her toes impatiently. Her hostile passive attitude became even more apparent when the child needed to put her clothes back on again. At this point the mother did not offer any help, leaving it entirely up to the doctor to give the child some assistance, and remaining defensively aloof at all times.

Verbally, however, this mother seemed to deny her negative attitude by talking about "what a terrible thing it was for a child to have so many injections." She was very consistent about bringing the patient to the clinic for her weekly visits, as if to say, "see what a good mother I am. I am doing all I can." In reality, her attitude towards the patient was overprotective and suppressive. For instance, she did not give the patient freedom to take part in normal play.

This mother's history showed that she was dominated quite similarly by her own mother who was a very demanding and controlling in various ways. For instance, she recalled that her own mother did not give her much freedom to date young men and blocked her father's plans to send her to a local business college which she had wanted to attend very much. Instead she was made to stay at home to help her mother until she finally married at age twenty-five when her older brother brought a man four years her senior to the family's home.

*Discussion:* This mother shows clearly some of the internal and external pressures which we believe are characteristic for this group of mothers. Having been raised by a domineering mother, she had not found the strength as an adult to resist the numerous requests for all kinds of help and favors from friends, relatives, and neighbors, but instead tried to please and placate every-

body in her environment by giving into all kinds of demands for help, only to feel angry and exasperated afterwards.

Outwardly she tried to deny how she really felt about being taken advantage of in such a manner. For instance, when asked at the time of her weekly interview, how she had been, she would murmur timidly and with a pinched smile, "Oh, I was a little busy," which might refer to nursing a sick neighbor or assisting a pregnant relative or being currently involved in some charitable project to add on to her ordinary family chores, which involved taking care of her husband and three children, as well as supervising her eighty-two-year-old mother's nursing care.

Such masochistic sacrifices of all kinds only masked the emotional struggle that went on underneath the surface. Actually these sacrifices weakened her for her primary task of raising her children, especially her youngest (asthmatic) child, with appropriate tolerance and patience.

Her constant exasperation with her many self-inflicted burdens lefts its mark. It seemed to account for her premature signs of aging, her shrinking posture, and what appeared to be a gradual withering away.

The young patient, on the other hand, appeared quite inhibited as far as natural expressive behavior was concerned. She consistently showed a rather blank facial expression that appeared to deny both her deep-rooted dependency needs for her mother and her negative feelings towards the same mother who suppressed this child's desire to be with other children and learn from them all kinds of things, such as to play and to fight and to make up again.

It seemed then, that the mother's impact upon the patient during her formative years may have been such that it inhibited her from spontaneously using speech, facial expression, or crying, to deal with her mother's behavior like most children would. Instead the patient may have most likely felt compelled to fall back on pre-verbal somatic expressions of her emotional conflicts over her dependency needs, culminating and exemplified by her severe asthmatic attacks which frequently necessitated trips to the emergency room of the hospital.

*Case Illustration No. 2*

Whereas the patient's mother in the above mentioned case complained that the patient's birth was an unwelcome event because it came too late in her life, at a time when she was less flexible, less tolerant, less capable to adapt herself to the never-ending needs of a growing child (in fact having a baby at her age made her feel grotesque and ridiculous, she implied) the mother in our second illustration complained that her baby had come too early and at an inopportune time.

*Patient Larry H., Age 6* is the older of two children; he has a sister age 2 who was born when the patient was almost 4 years old, at which time he already had had frequent asthmatic attack for about two years. At present the child seems to lack in spontaneity.

The patient's mother, a slender, attractive, neat, well poised, lower middle class negro woman conceived the child when she was 17 years old, about six months before she got married to a three-year older man, a high-school sweetheart, whom she described as athletic, ambitious and very appealing. The

mother, now 24 years of age, also leaves the immediate impression of being ambitious, well disciplined, and having relatively high goals for her family and herself as far as moving up vertically on the social-class structure is concerned. The doctors, when first discussing this couple emphasized "how cooperative and intelligent" these parents were in all their contacts with the clinic and the hospital. The young patient, by his relatively fixed immobile aloof behavior, differed markedly from those more outgoing children who had been given freedom by their mothers to run up and down the hall and observe, play or draw in the examination room, making one wonder about the degree and quality of maternal control, under which he lived at home.

After some initial reserve, the mother was able, beginning with the second interview to be as frank and open in our discussion as one could possibly hope, leaving the impression that study and treatment in a medical setting was something that she could identify with and this being a sentiment that was stronger than the frequent fear of mothers to be evaluated and finally blamed for their child's illness.

It was learned that the patient was a full term baby, that pregnancy, labor and delivery had been uneventful and had not caused her any undue physical discomfort. (Her subsequent experience with her second child had been similar). In response to my question, while taking the history, she explained that she "preferred not to nurse the baby" because she valued her work as the regular piano-player for her church and did not want to endanger her position there. We got the impression that even though this was volunteer work, it did give her a good sense of status in her group, which as we could perceive from all subsequent material we got, was something that she had badly needed all of her life.

Then, with a facial expression that betrayed how distasteful the subject was to her, she recalled that the baby had cried very very often during his first weeks and months of life, he didn't sleep too much but always wanted somebody to hold him. Always wanted somebody to hold him! She repeated this with a tone of resentment, as if to say "this baby was holding me down."

The baby reacted to this negative attitude by wanting to cling even more, biting clear through his nipples and developing into a finicky eater. He had frequent colds. Since Mrs. H.'s mother, who lived with them had full-time employment as a maid, the person who took care of the baby a good deal of the time during the day, was "'mother Jackson." This arrangement in turn made it possible for the mother to take a six months course at a local beauty school and to take on a job as beauty operator shortly after she finished her training. All this meant that the baby was handled by two women in addition to the short periods that his real mother could or wanted to spend with him. However the regular care provided by "mother Jackson" who is described as a warm affectionate person in her sixties, who had no children of her own, did seem to give the baby a good deal of support, just as she had given to the baby's mother during the latter's formative years. Mrs. H. thus was able to work and earn on a job that she likes, continue as the piano player of her church and did not have to get involved with the baby anymore than she cared to. Then, when the baby was about 2 years old, mother Jackson died suddenly of a paralytic stroke and shortly afterwards the baby developed a

skin rash and a few weeks after that, experienced his first asthmatic attack (a severe one) while spending the night in his mother's bed. She had to take the child to the emergency room around 2 a.m., a trip that she had to repeat many times since then, as at first attacks occurred every day.

Just as when her husband was out of town with his football team, when the baby was born, he was also away from the mother (serving with the Army in Alaska), when the child's first asthmatic attack occurred and regular visits to the Allergy Clinic had to be instituted by the resentful mother. The mother had to interrupt her employment until her husband was discharged by the Army and could take on a night job as an orderly, to free the mother to accept employment at a fashionable beauty parlor, which to her meant moving upwards as far as prestige was concerned. However this gratifying job had to be abandoned after only a few months, when Mrs. H. found herself unexpectedly pregnant with her second child, a girl, who was born in 1958. In line with her life-experience, specifically the aloof behavior of her father and the more acceptant attitude of her mother towards her, she has found it easier to identify with her daughter, whom she even nursed for a period of time, and towards whom she does not seem to feel resentful.

As a result of the above mentioned developments, the mother grudgingly had to reduce sharply her social and occupational activities, for instance is limited now to taking care of a few women in the neighborhood, who will come to her house to get their hair fixed.

With regard to the possible relationship between her son's behavior and his physical reaction on one hand and her way of handling him, she only developed a flicker of self-awareness. This was stimulated by her observation that the child during his first months of life, whe he had prolonged crying spells, responded favorably, for instance, cried less, slept better, when she switched him from a rigid feeding schedule that was convenient to her to feeding him on demand. Upon question, she was also able to tell me that she seemed to be able to influence favorably the length, frequency and intensity of his asthmatic attacks, by forcing herself to stay in the room with him, when he seemed to reach out for her and if he did have an actual asthmatic attack, by holding him grudgingly on her lap for as long as two hours or more, until he breathed easier.

To sum up then, what the experiences might have looked like from the child's point of view, one observes that he was taken care of in the beginning by three women, his mother who tried to avoid him, his maternal grandmother who is described as rather nervous, irritable and overprotective and until age 2 by mother Jackson, whose sudden death was bound to be interpreted by the young patient as a personal rejection by the one mother-figure, who evidently was more affectionate and consistent in caring for him than either of the other two women.

*Background Information:* The general reaction of the mother became understandable during subsequent interviews. She mentioned then that during their courtship her husband had attended college in Houston, planned on becoming a medical technician but had to interrupt this project when an unexpected pregnancy compelled him to get married and take on a job as an orderly at a local hospital to support his new family. Her husband reportedly took his

disappointment in his stride and is still preparing to return to college to follow out his original plan in the foreseeable future as he has been granted a scholarship since. The mother not only had high goals for her husband but also for herself personally. She recalled that during their courtship they had talked about how they could attend college together, walking hand in hand around the campus, where she had planned on preparing to become a teacher in the public school system, specializing in "Family Education and Homemaking." (Evidently trying to do in her occupation what she could not do in her previous life,) as will become understandable soon. Instead she found herself pregnant and had to drop all of her plans of becoming an educator.

The pregnancy and the coming of the child also had special significance to the mother in still another way. She came from a broken home, her father, a longshoreman, had left and divorced her mother when she was only two months old. She maintained contact with her father through frequent visits but noticed with chagrin that the father openly showed more interest in her 3 year older brother.

The fact that her mother showed some preference for her over the brother did not seem to make up for that, since the mother, holding employment as a maid, left her in the care of a neighbor whom she liked and whom she called "mother Jackson". As to her real mother she remarked: "We never felt real close". An example of this distance that existed between her mother and herself was the mother's lack of interest to prepare her adequately for life as an adult. For instance, menstrual bleeding at age 12 came to Mrs. H. as a shock, and when she asked her mother about it, the mother seemed greatly embarrassed and only told her: "You will find out in due time." She remembers herself generally as a sensitive, nervous child, who because of her mother's attitude thought that " all sex was something very dirty" and to use her words, "that having sexual intercourse would be the end of the world."

As it turned out, when, as a result of an unplanned pregnancy she had to get hastily married, drop all of her hopes and dreams of a professional career, especially meaningful for a Negro struggling for a greater acceptance in our society, and in a sense "the end of her world" and even forced her husband to give up college which happened to be the one feature about him and his relatively ambitious plans that attracted her most to him.

She recalled that she had never, in the past, (or even now) been particularly interested in her sexual relationship but rather afraid with her first child, now still feels resentful, when her husband wakes her up at night to insist on having intercourse "always at the wrong time." She does not reach a climax very often at present and very much against her will finds herself eight months pregnant with their third child.

When I asked her about whether she preferred to have a girl or a boy now, she said with emphasis, "I had all I wanted, a boy and a girl, that is all we can afford financially, but since I am pregnant again I hope this baby will be a girl." Girls, she added, are neat and pretty, boys are hard on their clothes and so hard to discipline in comparison with little girls.

*Discussion:* To return now to the history of the six year old young patient's first years of life, some of the following factors may also have a potential predisposing influence, as far as Mrs. H.'s relative incapacity for adequate

mothering was concerned. Mention was made of the fact that she herself would have preferred a more protective atmosphere such as provided in a college setting to gain status and prepare for a career as a high school teacher. My impression was that such a period of grace, as one might call it, might have given her an opportunity to work out and overcome the impact of life in a broken home, without genuinely capable, interested parents to love and guide her. She was unduly apprehensive of the area of sex and otherwise socially adequate in her adjustments.

Of late, as mentioned above, we have begun to add another step to our investigation which we consider of utmost diagnostic value; namely a visit to the homes of selected asthmatic patients who reside in Galveston County. This will be done in the hope of gaining a better understanding of the patients' situation in the family and the relationship with the parents.

*Home Visit:* By the time this home visit was made to get a more graphic picture of the patient and his family situation, which as mentioned above is part of our method of collecting relevant data, the patient's mother, Mrs. H. had given birth to a boy and not to a girl as she had hoped.

When we arrived, she showed us the baby boy with a resigned smile and made a strong attempt to look brave and happy. The content of our discussion with the members of the family who were at home (the patient, patient's mother, patient's younger sister, baby boy, and maternal grandmother) centered around allergic factors, such as dust, molds, and undesirable types of food which might stimulate asthmatic attacks in Larry, the patient.

While this discussion went on we observed that there were a good many photographs of the patient's younger baby sister around, showing her at various ages and in different surroundings, but there was only one small picture of Larry, the patient, tucked away in a corner.

During our talk with the mother and grandmother, the patient and his younger sister watched a children's program on television, and after the young girl had sat closely to the mother leaning tenderly against the mother's leg, the boy tried to do the same when his sister went out to show us some drawings she had made. At this point the mother instinctively withdrew her leg and the boy had to move away from her. There were other indications of the mother's unwittingly rejecting attitude towards this boy, the patient, who had come into her life too early and at such an inappropriate time.

*Case Illustration No. 3*

*Omar C., age 9.* This case is mentioned briefly because the patient is one of six children, none of whom has asthma. The interviews with the mother revealed that when she was pregnant with the patient (at age 40) her mother had become critically ill, resided in a small community almost 700 miles away. Since it was tradition in her family that it was the task of the only daughter in the family to come to the mother's help at time of illness, the

young patient's mother had felt in great conflict when the family put pressure on her to leave Galveston and come to her mother's help. She waited until the birth of the baby, (the patient) and left him when he was a little over ten days old in the care of his 17 year old sister. She remained with her mother for two weeks until the mother, herself, recognized the tension under which Mrs. C. lived and told her to "go home and take care of her baby." This Mrs. C. did, but since her mother died a few weeks later and the attitude of her not-too-understanding siblings remained reproachful, she and the patient got off to a bad start and the patient, during the first year of life, responded to the negative handling of his mother typically; first, with skin rashes and finally with asthmatic attacks, the severity of which has remained unabated.

### *Reaction to Traumatic Events*

In addition to asthmatic cases which could be traced back to the baby coming into the life of the mother too early, too late, or otherwise at an inopportune time, we also encountered a number of patients where the onset had not occurred during the first or second year of life but several years later when they responded to a very traumatic experience, first with a pronounced nervous reaction which eventually crystallized in a condition of severe and chronic asthma with frequent attacks necessitating trips to the emergency room of the hospital.

#### *Case Illustration A*

*Robert, E. R., age 11*, developed asthma shortly after he witnessed the death of his father as the result of a coronary thrombosis at age 8 (1957). Other symptoms appearing in response to the traumatic event were frequent urination and sleep disturbances.

Discussion with the mother showed that she was a rather rigid, conventional person who could not make up the loss of a genuine warm relationship he had experienced with his late father. Instead she reported about her boy's condition in cold, professional terms familiar to her from previous nurses training, and left the impression of an ambitious, hard-driving, perfectionistic mother who criticized the boy when he curiously touched medical instruments that were displayed on the table and made him say "excuse me" whenever he wanted to leave the room to join the other children who played freely in the hall.

#### *Case Illustration B*

*Walter Allen B., age 10.* This young Negro boy developed asthma at age 7 when he witnessed the accidental drowning of his two older brothers. He had been used by them as a messenger to secure permission from the father who was waiting in a truck for them and gave the permission for them to stay on in the water. Following the drowning and his being exposed to the sight of his dead brothers in the open coffin, he developed severe nervous reactions

marked by crying spells, nightmares, extreme restlessness which was handled by the family doctor with sedation. Severe asthmatic attacks which have persisted until the present time set in only a few weeks following the death of his two brothers.

The mother, when seen, left the impression of a rather unstable, impulsive person who, as was hinted by the father, had when first receiving the news of the death of the boys blamed the patient by implication for the drowning, although she has since reversed her opinion. Because of her rather rigid personality she was unable to help the young patient over the traumatic experience.

*Case Illustration C*

*Finally the situation of Ronald T. C., age 13*, should be mentioned. This patient had experienced severe asthma attacks since the first year of life. His symptoms related clearly to a disturbed mother-child relationship and because of the psychopathic personality of the mother a rather radical recommendation had to be made proposing that the young patient be physically separated and live away from his mother to safeguard his life at this time.

It is not clear whether the family will be able to follow out his medical recommendation.

## *Discussion*

Probably few experiences come to a child which are as devastating as asthma. Breathing is likened to the most primitive roots of fear and rage (the panting breath of the angry person is well known). In the midst of an asthmatic bout, what then must the child feel towards the adults, who stand helplessly around him, breathing freely themselves, yet are unable to bring significant relief to the child. The usual parental concern and increased attention which focusses upon the child after such an event, becomes almost a constant feature in the child's relationship with the mother. In particular this is frequently accompanied by secondary gains and stimulates the notoriously dependent clinging behavior, which characterizes so many asthmatic children.

Although hypersensitivity provides a basic mechanism for bronchial asthma, asthma may be called a disease of dependency and like other psychosomatic disorders has its origin in the mother-child relationship. The meaning and effect of the symptoms is to keep the asthmatic child as much as possible dependent on his mother and prevent his functioning as an independent individual. This is very much in contrast to the way in which a wise parent makes herself gradually unnecessary as the child grows stronger.

Much has been said about the factor of maternal rejection in

asthma. (2) Most observers have concluded that such attacks are precipitated by situations that threaten to separate the patient from his mother or some mother-figure, such as his wife, but in our experience the separation feared is not necessarily actual physical separation from the mother, but more frequently the real or imagined danger of some estrangement or a threatened loss of the mother's affection or approval. We felt that the concept of maternal rejection may have been too narrowly interpreted in the literature, considering the fact, that even the death of a mother is also felt as a direct rejection of the mother by the young child who does not yet comprehend what death means.

In line with this was encountered a variety of negative attitudes among the mothers. At one end of the scale was the physically ill mother, suffering from overweight and high blood pressure and obviously preoccupied with her own bodily impairment who was unable to respond to the advances of her child, thus unwittingly leaving him with the impression that she was not very much interested in him.

In contrast to this, there was the mother, who felt so negative towards her adopted son, that she always sent the adoptive father to the clinic with him, instead of coming herself like the other mothers. The father reported various details of her hostile behavior to this asthmatic Negro boy, such as reproaching him for the unconventional way of life that his natural mother had selected for herself. At the same time she would force him to write to this mother from whom he had been separated since he was two days old. Repeated interviews with this father, who was genuinely fond of the boy, gave him enough support to confront his wife with the meaning and impact of her rejecting behavior, with the result that the boy's asthmatic attacks have become a rare event.

It was our impression that any counseling or supportive therapy when used as a means of rehabilitation in asthmatic children should be wisely limited to careful environmental manipulation and showing and if possible demonstrating the connection between the occurrence of the symptoms and certain effects, such as anger, resentment, disappointment and fear, without any interpretation of specific conflicts. (The latter would be the task and area of dynamic psychiatry and should be reserved for treatment by a specialist in psychotherapy). The patient in such

supportive therapy may be encouraged to recognize his feelings and to express them during the interview as well as become generally more expressive and outgoing in his daily activities. This typically may involve reassuring an overprotective mother again and again that it is not only "all right" but even desirable for the asthmatic child to join his peers in active play, including taking part in trips to the swimming-pool or beach.

As far as the asthmatic child is concerned, the better he is able to recognize and to express appropriately the actual emotions behind the symtoms that give rise to them, the less need he will have for his symptoms. This may involve a discussion of how unhealthy it is "to give in when you know that you are right only to feel angry afterwards" and possibly express this anger in somatic symptoms. The mother may, if ready for such a topic, be given some awareness of the neurotic gain that the asthmatic child obtains from maintaining an unduly dependent relationship with the mother and how *undesirable* it is to continue to do for the child things that he by now can do for himself. It may be possible if a good therapeutic relationship has developed between the interviewer and the mother to confront her with the fact that her own neurotic satisfaction derived from doing things for the child and thus keeping him unduly dependent on her is actually unhealthy.

Since from supportive therapy often no more than temporary symptomatic relief and mitigation of the severity of the attacks can be expected, the most hopeful aspects of the problem presented by asthmatic children is in the area of prevention. An encouraging beginning has been made in several prenatal clinics, in work with pregnant women who have evidenced behavior and symptoms that would lead to the assumption that their relationships to the expected child would be conducive to a psychosomatic response in the child.

## *Summation*

This report is a description of an ongoing project at the University of Texas Medical Branch, centering around a pilot study of the interplay between mothers and their asthmatic children who were seen at the Allergy Clinic.

We attempted in each case to formulate what can be described

as a family-diagnosis. Because of the importance of the mother-child relationship, we made a particular effort to evaluate each of the mothers in the sample group on the basis of her personality and ego-strength, and in the light of both her current reality-situation and her past life history. In this connection we focussed on the "special pressures" under which all of these mothers were found to be living, to appreciate their impact on the mother's attitude towards her pregnancy and the baby after it arrived.

In other words, to make an adequate evaluation we went back to the period *prior* to the conception and birth of the asthmatic child because we believed that the mother's personality as shaped by her experience during her formative years in her parents' home had greatly influenced the quality and scope of her various mothering-activities.

We found that as far as the mother's subjective opinion was concerned, the asthmatic child had often come too early, too late or otherwise at an "inopportune time" in the mother's life. In those few cases where the child's asthmatic attacks had been preceded by a severe traumatic experience (such as witnessing the death of the father or siblings), the relationship with the mother was never a strong and stable one, but had been rather delicately balanced and was marked by an ambivalent attitude on the mother's part.

Many mothers in spite of the skill, sensitivity, and charm that they could demonstrate in a social situation or the smooth seemingly serene façade of maturity and maternal capacity that they appear to possess were usually quite unprepared for their task to raise an asthmatic child.

C. B. Chishold, a Canadian psychiatrist, observed in reaction to the family problems that had come to his attention, "We have learned to raise pigs, cows, and horses and even grow flowers and vegetables in ways that make them of greater service to mankind. If your son is going to raise pigs for a living, he goes to college for three or four years to study under experienced teachers, but if he is merely raising children, he commonly learns nothing, nor appreciates even dimly that there is that he has to learn. Surely the raising of children is greatly more important than the raising of pigs."

The psychiatric nurse is one example of a woman given what might be termed semi-maternal care to a youngster or what one

may call an adult-child. Drs. Harris and Ford of the University of Texas Medical Branch observed quite early "That the attitude of the average nurse toward a psychiatric patient is the same as that of any untrained individual." The authors add that only "Experience teachers her to have a more sympathetic attitude toward the complaints of the patient. . . . In other words, she has an opportunity (through specialized training) to acquire the much needed psychiatric viewpoint." (10)

There is little doubt that one of the main functions of the family is the regulation of balances of the personality of the adult members, especially, since the mother's gratification in satisfying her child's needs or of frustration over her failure to do so often affects her emotional life and reciprocally that of the child. The development of the child or adolescent most always influences the developmental processes of the parent—in fact it determines whether or not the relationship with the satisfying child helps to resolve the remnants of the developmental conflicts of the parent or—whether anxiety and aggression stimulated by becoming and having to function as a parent now, actually activates old conflicts in the parents, especially in the mother, and motivates new disturbances.

Since the successful stabilization of the adult personality in the mother usually depends on her having had a successful experience in raising her child, it becomes evident how valuable and important it is to facilitate such final stabilization of the adult personalities in the parent with its beneficial consequences affecting the children available by focusing treatment not only on the asthmatic patient but also on the mother and the family as a whole. For this and other reasons, we felt that the role played by "special pressures" affecting the mother was of major importance.

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# ESSENTIAL OILS, PLANT EXTRACTS AND CHEMICALS FROM PLANTS IN CANCER CHEMOTHERAPY

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Many essential oils have been used for medicinal purposes in the past, and their long use in perfumes is well-known. The present report deals with results of screening for anti-tumor effects of some of the essential oils, as well as of plant extracts prepared in our laboratory.

## *Materials and Methods*

Essential oils from plant sources were used in these studies rather than synthetic oils which have replaced many of the essential oils in industry. The majority of the plant extracts in this report were made from common vegetables and ornamentals. All vegetables were secured from a local store, and the varieties are not known. Ornamentals were secured from Vaughan's Seed Co., Chicago, Ill. Chemicals were sent us with such data as is available about their chemical properties and physiological effects (3, 14).

All the material was tested against tumors transplanted in eggs and mice. The methods used have been described (10, 11, 13).

For tests against tumors cultivated in mice, a dba mouse mammary carcinoma, known as the RC mammary adenocarcinoma, was used. This tumor has been used for screening in other laboratories (9). Mice with transplants of this tumor were given one injection of the test material at or near the tolerated dosage. The material was injected 72 hours after the tumor was implanted. The effect of the material was evaluated on the basis of the average weight of the tumors from the treated as compared with the average weight of tumors in the control group for a 9-day test period.

Yolk sacs of embryonated eggs were inoculated through the air sac on the 4th day of incubation with a saline suspension of dispersed tumor tissue. A $C_3H$ mouse mammary carcinoma especially adapted to grow by this type of cultivation was used.

The test extract was injected over the area vasculosa of the tumor bearing eggs on the 8th to 12th day of incubation, or 4 to 8 days after eggs were inoculated with the mouse tumor. The effect of the material was assessed on weight differences in tumors and embryos of the experimental eggs as compared with the controls on the 13th day of incubation.

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Essential oils were obtained from commercial suppliers and by steam distillation of plants native to Texas. Oils from *Monarda fistulosa* and *Pectis texana*, plants native to Texas, have been reported (1). A surface active agent was used with the oils to get them into emulsions. The most satisfactory emulsions were obtained with Aerosol TR (2).

Vegetables and ornamental bulbs were pressed in a Carver hydraulic laboratory press. The expressed liquid was immediately freeze-dried and the dried material used in tests. The remaining marc was then extracted with $H_2O$ and then 95% ethanol. In general, pressing required more raw material than we had anticipated to provide sufficient quantities of freeze-dried material for test purposes.

Acetone and petroleum ether were used on the pressed material of Dutch Iris. *Chelidonium majus* and *Chrysonthamnus nauseous* were dried and extracted with $H_2O$ and then 95% ethanol. The seeds of *Daubentonia longifolia* were extracted with $H_2O$ only. Expressed oil from Sweet Almond was used for test purposes. Crocus bulbs gave very little material either by pressing or extraction. Jonathan apples provided a sufficient quantity of expressed liquid but small amounts of dried material, and subsequent extraction yielded little material.

Small amounts of streptomycin were added to the extracts and some essential oils just before injection for control of bacteria. The essential oils were generally effective anti-bacterial agents.

## *Results*

The results are summarized in Tables 1, 2, and 3. Beta Thujaplicin and American Cedarwood Oil are of interest because they inhibited tumors cultivated both in the egg and mouse. Results with vegetables and ornamental bulbs gave very little encouragement. Increasing the time between injection and harvesting of tumors cultivated in eggs did not appreciably increase the effectiveness of the test material. The 24-hour test period appears to be the most effective.

## *Discussion*

In general, essential oils are very toxic to living material and on that basis most of them appear to be of little or no value as chemotherapeutic agents. In this screening project, American Cedarweed Oil and Beta Thujaplicin, a plant product obtained from Western Red Cedar, were the only plant fractions which merited further tests. Both these extracts inhibited the growth of the RC mammary tumor cultivated in mice and a $C_3H$ mammary tumor grown in eggs. In the tests with egg-cultivated tumor tissue the tumor was inhibited in growth with little or no effect on the

TABLE 1

*The effect of essential oils, chemicals and plant extracts on the growth of tumor and embryo of tumor-bearing eggs, 24 to 120-hour test periods*

| Plant material | No. expts. | No. eggs | Dosage | Survival, Control = 100 | Aver. Growth for Test Period, Control = 100 Embryo | Tumor |
|---|---|---|---|---|---|---|
| **24 HOUR TEST PERIOD** | | | | | | |
| Carrots | 3 | 142 | 1 mg + .02 mg + Strept.[3] | 99 | 83 | 53 |
| Oil of Sweet Birch | 2 | 87 | .01 ml + .0025 mg TR[4] | 99 | 46 | 8 |
| American Cedarwood Oil | 2 | 89 | .01 ml + .0025 mg TR | 98 | 92 | 60 |
| Oil of Monarda dispersa | 2 | 89 | .006 ml + .02 mg Strept., + Trace TR | 101 | 94 | 50 |
| Pistacia chinensis[1] Alcohol ext. of leaves | 2 | 90 | .1 ml | 87 | 107 | 17 |
| Pistacia lentiscus[1] Alcohol ext. of leaves | 3 | 130 | .1 ml | 90 | 120 | 40 |
| Oil of Sage | 3 | 135 | .0025 ml + .00625 mg TR | 98 | 87 | 29 |
| Thujaplicin[2] | 2 | 88 | .003 ml + Trace TR | 83 | 117 | 25 |
| Beta Thujaplicin[2] | 4 | 140 | .4 to .56 mg + Trace TR | 99 | 117 | 26 |
| TR | 2 | 90 | 2 mg | 55 | 107 | 113 |
| | 1 | 45 | 0.2 mg | 100 | 120 | 103 |
| **72 HOUR TEST PERIOD** | | | | | | |
| American Lemon Oil, U. S. P., Expressed | 2 | 89 | .01 ml + .0025 mg TR | 100 | 159 | 32 |
| **120 HOUR TEST PERIOD** | | | | | | |
| Anise Oil, U. S. P. | 2 | 89 | .01 ml + .0025 mg TR | 73 | 105 | 21 |

[1] Contributed by U. S. Plant Introduction Station, Chico, California.
[2] Contributed by Crown Zellerbach Corp., Camas, Washington.
[3] Dihydrostreptomycin Sulfate U. S. P.
[4] Aerosol TR.

TABLE 2

*The effect of subcutaneous injections of an essential oil and a chemical in DBA mice bearing transplants of RC mammary carcinoma, 9-day test period*

| Plant material | No. expts. | No. mice | No. inj. | Dosage per inj. | Body weight at autopsy, control = 100 | Tumor growth at autopsy, control = 100 |
|---|---|---|---|---|---|---|
| American Cedarwood Oil | 3 | 27 | 1 | .01 ml + .0025 mg TR[2] | 99 | 48 |
| Beta Thujaplicin[1] | 2 | 18 | 1 | .37 to .46 mg + Trace TR[2] | 97 | 44 |

[1] Contributed by Crown Zellerbach Corp., Camas, Washington.
[2] Aerosol TR.

TABLE 3

*Essential oils, plants and plant products with little or no inhibition of egg or mouse cultivated tumors*

Essential Oils

Anethole, N. F.
Bay oil, N. F.
Benzaldehyde, N. F.
Citral, M, M and R
Citronella Oil, Ceylon
Clove Oil, U. S. P.
Eucalyptus Oil, N. F., distilled
Fennel Oil
Lavender Flowers Oil, U. S. P.
Oil of Birch Tar, Rectified, N. F.
Oil of Sweet Birch, U. S. P., Southern
Oil of Bergamot, N. F.
Oil of Bitter Almond, N. F.
Oil of Cade
Oil of Star Anise, U. S. P.
Oil of Cedarwood
Oil of Mountain Laurel (Oil of Ambellularia californica, Nutt) (1)

Essential Oils were obtained from the following plants native to Texas

Anethum Graveolens
Artemisia mexicana
Chrysactinia mexicana
Hedeoma reverchonii
Koellia mutica
Matricaria chamomila
Mentha alopecuroides
Monarda dispersa (non-phenolic portion)
Monarda punctata (non-phenolic portion)
Monarda punctata
Ocimum basilicum
Origanum vulgare (wild marjoram)
Pectis texana

Plants tested

Edible

Apple, var. Jonathan
Almond, sweet, nut
Beet, root and leaves
Cabbage, head
Lettuce, head
Pumpkin, flesh of fruit

Ornamentals
- Crocus bulb
- Dutch Iris, var. Wedgewood
- Gladiolus corms
- Tulip bulb, var. Albright

Other plants
- Corn, cob (2)
- Chelidonium majus (Great Celandine Herb)
- Chrysothamnus nauseosus (3)
- Leptotaemia dissecta, roots
- Argemone hispida (4)
- Daubentonia longifolia, (Sesbania Cavanillesii), seeds
- Pistacia chinensis, immature fruit and stems (4)
- Pistacia lentiscus, stems (4)
- Pistacia terebenthus, immature fruit, leaves and stems (4)

Chemicals

Quercetin (5)
Thujaplicin (6)

(1) Contributed by Fritzsche Bros., Inc., N.Y., N.Y.
(2) Contributed by The Andersons, Maumee, Ohio.
(3) Contributed by Dr. Jos. H. Robertson, Univ. of Arizona, Reno, Nevada.
(4) Contributed by U.S. Plant Introduction Station, Chico, California.
(5) Contributed by Weyerhaeuser Timber Co., Longview, Washington.
(6) Contributed by Crown Zellerbach Corp., Camas, Washington.

growth of the associated embryos. Beta Thujaplicin appeared to be better in this respect than the Cedarwood Oil. More experiments, especially those involving mice with naturally occurring tumors, will be necessary to establish the cancer therapy values of these materials. All the material listed in Table 1 was tested in the mouse, but only two inhibited tumor growth.

Again, as in past investigations of the effects of plant fractions on tumor growth, the data obtained with these essential oils indicate the usefulness of the plant kingdom as a source of biologically active substances. Such screening tests reveal extracts with a wide range of activity and a small percentage of these manifest tumor growth inhibiting qualities (4, 5, 6, 7, 8, 12, 13).

## *Summary*

A total of 40 essential oils and extracts from 22 plants were tested for their effect on tumor growth. Each extract was evaluated on the basis of its effect on both mouse and egg cultivated mouse mammary tumor tissue.

American Cedarwood Oil and Beta Thujaplicin, an extract

from Western Red Cedar, inhibited the growth of egg cultivated tumor tissue 40 and 74 per cent respectively with little or no effect on the growth of the associated embryos. Both of these extracts inhibited the growth of tumor transplanted in mice slightly more than 50 per cent.

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# EGG-LAYING CAPACITY OF THE SNAIL *BULINUS TRUNCATUS* IN RELATION TO INFECTION WITH *SCHISTOSOMA HAEMATOBIUM*

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Although the effect of digenetic trematodes on their snail hosts is variable, it is generally accepted that infected snails have a higher death rate and shorter life span than those uninfected. Moreover, the reproductive capacity of infected snails is affected. This is particularly important in view of the fact that the control of the molluscan host seems to be the most promising method in attempts to control bilharziasis.

The data of this paper represent laboratory observations on the egg production of uninfected *Bulinus truncatus* and those infected with *Schistosoma haemotobium*, the causative agent of urinary bilharziasis in Iraq. A quantitative estimation is given of the effect schistosome infection had on egg production of the snails, both in terms of the average number of eggs laid per clutch and the rate of oviposition.

## *Materials and Methods*

The *B. truncatus* were collected on the same day from a ditch in the vicinity of Baghdad, Iraq. Since it has been demonstrated that in the laboratory *B. truncatus* commence egg-laying when the snails are 6.5–7 mm long (10), snails measuring 8.5–10 mm long were selected and individually isolated in small dishes containing 30 ml of non-aerated tap water. The water was changed daily and either boiled lettuce or spinach was used as food. The number of egg clutches and eggs per clutch produced daily by 6 naturally infected and 5 uninfected snails was followed in the laboratory for 33 days. All of the infected snails were shedding *Schistosoma haemotobium* cercariae both at the beginning and end of the experiment. The water temperature during the period of observation was 15.5–25.5° C. The dishes remained in the same laboratory location throughout the experiment.

[a] This study was made while with the WHO Bilharziasis Control Project in Iraq. The contribution of J. A. Glasenapp, Department of Preventive Medicine and Public Health, The University of Texas Medical Branch, Galveston, Texas, in the statistical analysis of the data is appreciated.

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## *Results*

A comparison of the egg-laying capacity of the two series of snails is given in Table 1.

TABLE 1

*Comparison by analysis of variance of egg-laying capacity of* B. truncatus *snails, infected and uninfected with* S. haematobium *cercariae*

| Egg production | | Snails: Infected | Snails: Non-infected | p value |
|---|---|---|---|---|
| Eggs per | mean | 4.7 | 15.1 | $<0.001$ |
| clutch | range | 0–7.2 | 12.9–16.0 | |
| Clutches per | mean | 0.24 | 0.59 | $<0.005$ |
| snail per day | range | 0–0.58 | 0.39–0.70 | |
| Eggs per | mean | 1.1 | 8.9 | $<0.001$ |
| snail per day | range | 0–4.2 | 5.1–11.2 | |

There was about a 3-fold reduction in the mean eggs laid per clutch, more than a 2-fold reduction in the mean clutches laid per snail per day, and an 8-fold reduction in the mean eggs laid per snail per day in the infected group as compared with the non-infected snails. A statistical analysis of the data resulted in the observed differences being highly significant ($p<0.005$).

The question arose as to whether all degrees of infection would significantly decrease egg-producing capacity. Because of the possible existence of various degrees of infection in the snails, it was decided to create a synthetic sample, with a mean equal to the highest actual value of eggs per day and clutches per snail per day, and a variance of these new values equal to the actual sample. To accomplish this, the difference between the actual mean and the maximum sample point was added to each sample point. An analysis of variance showed the mean of the synthetic sample to be significantly lower than the mean of the uninfected sample ($p<0.005$). If it is assumed that reduction in egg-producing capacity is an inverse function of a degree of infection, and a statistically significant difference results from the above comparison, then certainly any means lower than the synthetic sample would also be significant.

## *Discussion*

It is indicated by these results that, in the laboratory, and in

the case of *B. truncatus* with active infections of *S. haematobium*, egg production was reduced to a significant degree, and that schistosome infection had a greater effect on the mean eggs laid per clutch than on the mean ovipositions per snail per day. Although one of the infected snails did not produce any eggs during the 33-day period, it did, nevertheless, deposit 4 empty egg cases. Another infected snail laid no clutches or eggs during the 33-day period. This suggests that heavy infections of *S. haematobium* may preclude deposition of snail eggs for over a month.

Reviews of the effect of trematode infections on snails have been given by Michelson (9) and Abdel Malek (2). Although the physiological and morphological effect is variable among various groups of flukes, schistosomes appear to reduce snail reproduction capacity.

Laboratory studies on *Oncomelania quadrasi* by Pesigan *et al.* (13) have shown that egg production in the female snail is impaired by parasitism with *Schistosoma japonicum*. They found that there was a 5-fold reduction in the average number of eggs laid per infected female snail per day compared to egg production of uninfected female snails. Moreover, they indicated that the laboratory observations were indicative of field situations, since when field data of 18 months were summarized, it was found that the proportion of young in the population was inversely related to the infection rate.

Infection of snails with *Schistosoma mansoni* also appears to have an effect on snail reproduction. Brumpt (5) observed fewer eggs being laid by *Australorbis glabratus* infected with *S. mansoni*. Coelho (6), also working with *S. mansoni* in the same snail host, demonstrated that infection reduced significantly both the numbers of eggs laid per clutch and egg clutches per snail. In *S. mansoni* infections of *Biomphalaria boissyi* Abdel Malek (1) observed the number of ova in the acini of snail ovotestes to be reduced.

It is of interest to note that not only do fully developed schistosome infections reduce the egg-laying capacity of the snail hosts, but also shorten the life span of the snail, (3, 7, 14) make the snail more susceptible to molluscicides (12), more susceptible during drying conditions (11, 4, 5), and interfere with the nutrition of the snail (16, 2). It would seem accurate to state

that the infection of snails with schistosomes places them in an adverse position in respect to both survival and sensitivity to control measures. However, snail populations re-establish themselves quickly from a few progeny (8), in certain areas the infection rate is low (15), and snails have the ability to resume normal reproductive activities after loss of infection (6). Therefore, a closer look at this suggestion does not permit much optimism. Nevertheless, the effect of infection on snail progeny might be of considerable value if threshold survival values of snails could be established both experimentally and in the field. To date, little of this has been accomplished.

*Summary*

The egg-laying of similar numbers and size ranges of *Bulinus truncatus* snails, both naturally infected and non-infected with cercariae of *Schistosoma haematobium*, was followed in the laboratory for over a month. There was a significant reduction in the average number of eggs laid per snail per day in the infected snails as compared to the uninfected ones. The schistosome infection had a greater effect on the average numbers of eggs laid per clutch than on the rate of oviposition. This study supports the view that fully developed schistosome infections reduce the reproductive capacity of the snail hosts.

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# ELECTROENCEPHALOGRAPHIC STUDIES IN PHYSICAL FATIGUE[a]

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## *Introduction*

Since physical fatigue involves relatively profound changes in the body chemistry and metabolic processes as well as a number of subjective changes in the individual, it is postulated that the EEG will be altered from that obtained during the resting state. Fatigue has been variously described in the literature and texts (Bartley and Chute, 1947, Floyd and Welford, 1953). Regardless of attempts to define fatigue, it is probably true that there are physiologic differences in the state of fatigue from different causes. The condition of the body in acute physical exhaustion is most surely different from that in prolonged mental effort, and on the basis of this study the EEG seen after physical fatigue is not the same as that observed after mental exertion.

Electroencephalographic studies in mental fatigue and combinations of mental and physical fatigue have been numerous (Gruttner and Bonkalo, 1940; Barnes and Brieger, 1946; Armington and Mitnick, 1950). In our study the EEG changes observed after physical work with very little mental effort on the part of the subject, gave results different from those found by previous workers whose studies were limited primarily to mental fatigue.

## *Materials and Methods*

These studies were carried out on apparently normal medical students and staff members varying in age from 22 to 36 years. Equipment consisted of a four channel Grass Model III D Electroencephalograph using muscle filters and operated at a paper speed of 30 millimeters per second with a calibration of one centimeter for 50 microvolts. To produce a state of physical fatigue an A. R. Young Treadmill was utilized which was electrically operated at a speed

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of three miles per hour and equipped with a hydraulic jack calibrated in per cent slope in order that the angle of the walking subject might be adjusted.

A control record of EEG was made of each well-rested subject between 8 a.m. and 10 a.m. with the subject sitting in an arm chair and the feet elevated for comfort. Bipolar recordings were made from six electrodes placed on the scalp in the frontal, central, and occipital positions on each side of the head using the two ear lobes as ground leads. In the area of electrode placement the hair was clipped and the area thoroughly cleaned with acetone; then standard Grass silver disk electrodes were secured in place by betonite paste, collodion, and gauze to prevent their becoming loose during exercise. Control records of ten minutes were taken during which the subject periodically opened and closed the eyes to note alpha blocking, and at the end of the control record the subject was requested to hyperventilate for two minutes to observe any evidence of abnormality.

The exercise was begun in the same room within 15–30 minutes following the control record. During exercise, records were made of the changes in per cent slope, heart rate, blood pressure, respiratory rate, time and degree of sweating, and appearance of subjective fatigue. No attempt was made to record the EEG while the person was on the treadmill. While the subject was walking in the air-conditioned room at a temperature of 72–74° F., the slope of the treadmill was raised gradually to prevent muscle cramp, so that at the end of 50 minutes walking the slope was at 12%, and this was increased 1% each five minute period thereafter.

Subjects were instructed to walk until they felt they were unable to continue. Times from beginning to the point of subjective physical fatigue varied from 35 minutes (4 subjects) to 70 minutes (2 subjects), with the majority of individuals continuing for an average of 50 minutes. From 5 to 15 minutes prior to the end of exercise the subjects were at a maximum heart rate of 185 to 210 per minute (see Figure 1) with an increase in blood pressure to an average of 150/30 mm Hg and a respiratory rate of 30 to 48 per minute. At the point of fatigue, all were sweating profusely and complained of general tiredness, and their walk was noticeably labored and unsteady.

Within 2–3 minutes after stopping exercise, the subjects' EEG was taken for a period of 15 minutes, followed by 10 minutes rest at which time he was given a soft drink and allowed to talk and move about. Following this another EEG record was made and continued until the subject's pattern returned to the pattern observed prior to fatigue.

Each record was carefully examined before and after exercise using the method of Davis (1941) to calculate the alpha index. In this procedure the number of centimeters of alpha activity in 100 centimeters of record is equal to the alpha index, and arbitrarily only series of waves three or more in sequence at a frequency of 8–13 per second and amplitudes of 15 microvolts or more (modified from the Davis method of minimum amplitude of only 7 microvolts) are counted as alpha patterns. Detailed analysis of the frequency spectrum as proposed by Engel, *et al* (1944) was not accomplished; however, the range of frequency of the basic alpha pattern was noted before and after fatigue. The maximum amplitude is reported as the amplitude of at least five of the highest waves in the 100 centimeters of record examined.

*Results*

In all 16 of the subjects used in this study there was a significant change in the appearance of the EEG after fatigue. These changes occurred from immediately after the fatigue records were begun to four and sometimes as long as 10 minutes following

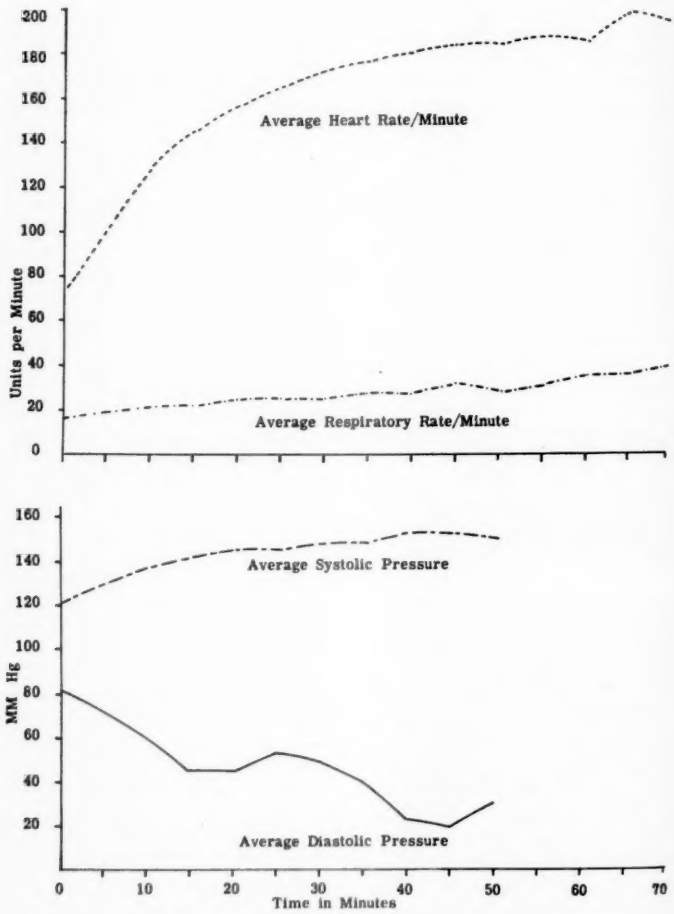


FIG. 1

exercise. The most marked difference in the records was an increase in the alpha index (per cent time alpha) which was more dramatically demonstrated in those subjects with only a moderate amount of alpha prior to physical fatigue. In some cases there was a significant increase (never a decrease) in the maximum amplitude of the waves. No significant change was noted in the range of alpha frequency after fatigue as compared with the control record. Table 1 is a resume of the results of the 16 subjects tested.

It can be seen graphically in Figure 2 and also in Table 2 that the increase in per cent time alpha is demonstrated best in the frontal to central bipolar leads, and those records with an increase of 5% or more above the control alpha index showed an average increase of 22–28% more alpha activity. The central to occipital bipolar leads showed slightly less increase of 17–19% more alpha activity. It will be noted in Table 2 that in all subjects tested there was an increase in the alpha index greater than 5% in at least one channel of the EEG record, and in all but one subject the increase was bilateral in two or more channels. Nine of the subjects showed a bilateral increase in alpha greater than 5% in the frontal region, and 11 had more than 5% increase bilaterally in the occipital regions.

As it is noted in Table 3 and figure 2, the average increase in amplitude varied from 15 microvolts in the right occipital region to 19 microvolts in the right frontal region with an average of 16 microvolts increase on the left. In eleven of the subjects with bilateral increase in amplitude, six showed this in the frontal region and five in the occipital region. Unilateral increase was observed on the right in three subjects and on the left in three other subjects.

Representative samples from the records of three subjects are shown in figure 3. In general, the EEG following fatigue was more regular in appearance, with more typical alpha patterns and less low voltage fast, irregular activity. On gross examination, the overall amplitude of alpha did not appear to increase after fatigue, and it was the increase in amount of alpha and regularity of the records which appeared most significant. In the one subject with no alpha activity in the control record, there was very little alpha after fatigue (10% in the occipital region) and this was irregular with superimposed beta activity.

The increase in alpha activity was observed to persist for 5 to

TABLE 1

*Per cent alpha and maximum amplitude for each subject before and after exercise. Bipolar leads from the right and left frontal, central, and occipital areas are used.*

| Subject | LF to LC | | LC to LO | | RF to RC | | RC to RO | |
|---|---|---|---|---|---|---|---|---|
| | Rest | Fatigue | Rest | Fatigue | Rest | Fatigue | Rest | Fatigue |
| 1. | 8% | 21% | 85% | 88% | 8% | 32% | 84% | 93% |
| | 20uV | 20uV | 40uV | 40uV | 20uV | 30uV | 40uV | 40uV |
| 2. | 38 | 60 | 95 | 99 | 26 | 30 | 88 | 88 |
| | 30 | 40 | 75 | 80 | 20 | 20 | 50 | 50 |
| 3. | 52 | 79 | 88 | 91 | 5 | 40 | 66 | 82 |
| | 40 | 50 | 75 | 75 | 20 | 35 | 65 | 65 |
| 4. | 15 | 33 | 78 | 94 | 5 | 39 | 94 | 97 |
| | 35 | 35 | 65 | 65 | 20 | 25 | 65 | 65 |
| 5. | 5 | 5 | 66 | 85 | 5 | 5 | 58 | 79 |
| | 25 | 25 | 55 | 75 | 20 | 20 | 40 | 50 |
| 6. | 6 | 30 | 57 | 82 | 1 | 1 | 60 | 85 |
| | 20 | 35 | 45 | 65 | 10 | 10 | 45 | 65 |
| 7. | 5 | 15 | 61 | 80 | 5 | 15 | 66 | 85 |
| | 35 | 60 | 75 | 75 | 20 | 60 | 75 | 90 |
| 8. | 61 | 82 | 94 | 99 | 70 | 88 | 98 | 99 |
| | 50 | 85 | 90 | 110 | 40 | 60 | 90 | 90 |
| 9. | 1 | 1 | 15 | 33 | 1 | 1 | 15 | 35 |
| | 10 | 10 | 25 | 50 | 10 | 10 | 25 | 50 |
| 10. | 5 | 20 | 55 | 73 | 5 | 10 | 60 | 80 |
| | 25 | 40 | 60 | 60 | 25 | 40 | 40 | 40 |
| 11. | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 |
| | 5 | 5 | 5 | 25 | 5 | 5 | 5 | 25 |
| 12. | 1 | 10 | 20 | 41 | 1 | 5 | 15 | 40 |
| | 10 | 20 | 20 | 40 | 10 | 20 | 20 | 40 |
| 13. | 36 | 65 | 88 | 99 | 18 | 38 | 90 | 99 |
| | 25 | 40 | 70 | 75 | 25 | 25 | 70 | 70 |
| 14. | 64 | 82 | 85 | 96 | 25 | 63 | 82 | 97 |
| | 40 | 50 | 120 | 120 | 25 | 50 | 75 | 80 |
| 15. | 12 | 52 | 45 | 90 | 21 | 58 | 72 | 89 |
| | 20 | 20 | 40 | 50 | 20 | 20 | 40 | 40 |
| 16. | 22 | 66 | 55 | 79 | 12 | 49 | 70 | 81 |
| | 30 | 30 | 30 | 30 | 20 | 20 | 45 | 50 |

10 minutes, and then progressively decline to approximately the same alpha index as the control record at the end of about 30–40 minutes after exercise. In some cases where the appearance of the increased alpha index was delayed, this was due to the inability to analyze the EEG record because of irregularities from muscle potentials and heavy breathing of the subject.

*Discussion*

The changes observed in the EEG in this study were striking and very different from those changes reported in conditions of fatigue which were caused by other than simple physical exhaustion. The conditions under which our subjects worked were those requiring the least of any effort other than physical, and indeed

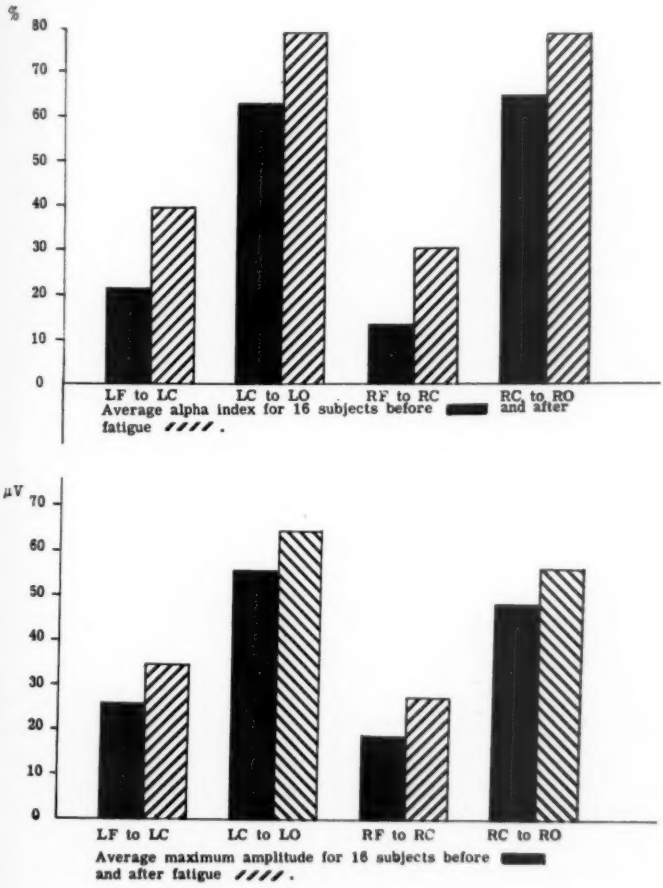


FIG. 2

TABLE 2

*Increase in per cent alpha activity for each subject after exercise*

| Subject | L Frontal to L Central | L Central to L Occipital | R Frontal to R Central | R Central to R Occipital |
|---|---|---|---|---|
| 1. | 13% | 3% | 24% | 9% |
| 2. | 22 | 4 | 4 | 0 |
| 3. | 27 | 3 | 35 | 16 |
| 4. | 18 | 16 | 34 | 3 |
| 5. | 0 | 19 | 0 | 21 |
| 6. | 24 | 25 | 0 | 15 |
| 7. | 10 | 19 | 10 | 19 |
| 8. | 21 | 5 | 18 | 1 |
| 9. | 0 | 18 | 0 | 20 |
| 10. | 15 | 18 | 5 | 20 |
| 11. | 0 | 10 | 0 | 10 |
| 12. | 9 | 21 | 4 | 25 |
| 13. | 29 | 11 | 20 | 9 |
| 14. | 18 | 11 | 38 | 15 |
| 15. | 40 | 45 | 37 | 27 |
| 16. | 44 | 24 | 37 | 11 |
| Avg. increase | 18% | 16% | 17% | 14% |
| Avg. of per cent inc. > 5% | 22% | 19% | 28% | 17% |
| No. with inc. > 5% | 13 | 12 | 9 | 13 |

Total showing some increase in per cent alpha = 16 or 100%

| | | |
|---|---|---|
| No. with increase per cent alpha >5% | all channels | 5 subjects |
| | 3 channels | 6 |
| | 2 channels | 4 |
| | 1 channel | 1 |
| | | 16 |
| No subjects with bilateral increase >5% | frontal | 9 |
| | occipital | 11 |
| No. subjects with unilateral increase >5% | right | 0 |
| | left | 1 |

it became an almost automatic and mentally effortless procedure to walk on the constant speed treadmill. It is probably true that none of the people tested were in a state of absolute exhaustion or collapse, however it was felt that a plateau was reached wherein the subject was tired and further effort was disagreeable and in some instances painful. In the subjective analysis of the person tested he was in a state of fatigue.

Recent work by Beaussart *et al.* (1959), in which EEGS of boxers before and after fights were compared, showed results somewhat similar to ours. An increase in amplitude and amount of alpha was found in a significant number of boxers after a contest. They could find no correlation between the changes observed and the degree of trauma incurred during the fight. It could well be that these changes observed by Beaussart and coworkers were the result of physical fatigue and/or mental stress since they found that trauma to the brain was not significant in the EEG findings.

TABLE 3

*Increase in maximum amplitude for each subject after exercise*

| Subject | L Frontal to L Central | L Central to L Occipital | R Frontal to R Central | R Central to R Occipital |
|---|---|---|---|---|
| 1. | 0uV | 0uV | 10uV | 0uV |
| 2. | 10 | 5 | 0 | 0 |
| 3. | 10 | 0 | 15 | 0 |
| 4. | 0 | 0 | 5 | 0 |
| 5. | 0 | 20 | 0 | 10 |
| 6. | 15 | 20 | 0 | 20 |
| 7. | 25 | 0 | 40 | 15 |
| 8. | 35 | 20 | 20 | 0 |
| 9. | 0 | 25 | 0 | 25 |
| 10. | 15 | 0 | 15 | 0 |
| 11. | 0 | 20 | 0 | 20 |
| 12. | 10 | 20 | 10 | 20 |
| 13. | 15 | 5 | 0 | 0 |
| 14. | 10 | 0 | 25 | 5 |
| 15. | 0 | 10 | 0 | 0 |
| 16. | 0 | 0 | 0 | 5 |
| Avg. those with inc. | 16uV | 16uV | 19uV | 15uV |

| | |
|---|---|
| No. subjects with increase in all channels | 1 |
| in 3 channels | 3 |
| in 2 channels | 8 |
| in 1 channel | 4 |
| total | 16 |
| No. subjects with bilateral increase frontal | 6 |
| occipital | 5 |
| Unilateral increase right | 3 |
| Unilateral increase left | 3 |

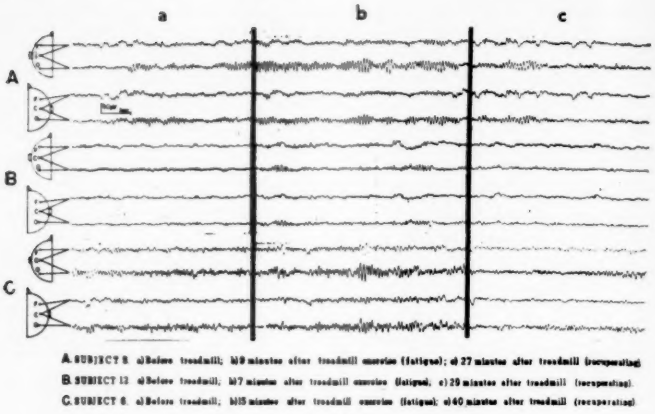


FIG. 3

Immediately following physical fatigue it appears that our subjects were in that "median" state of consciousness as described by Lindsley (edited by Jasper 1958) in which the ascending reticular activating system is exerting something less than its maximum influence on the cortex, and according to Magoun (1958) the cortical excitability in these subjects is somewhat diminished. The metabolic imbalance incurred in physical fatigue also might contribute to the changes observed in the EEG, although we do not know the extent of this influence. The increase in amplitude shown in Figure 3 and in Table 3 could be explained by the increase in synchronous activity of the brain during recuperation. The larger increase in alpha index in the frontal regions of most subjects would be indicative of a generalized spread of synchronous activity throughout the brain.

Assuming that alpha rhythm is a rhythm of rest (Faure, 1951), which may be blocked by attention (Jasper, 1947), and that it is a rhythm which maintains the individual in an optimum state of preparedness to receive incoming stimuli (Haussman, 1958), it can be postulated that the alpha rhythm increase as shown in Figure 3 and in Table 2 indicates that after physical fatigue the subject is less attentive to incoming sensory stimuli, and the awareness of his surroundings is not so acute. In most subjects complete lack of alpha in the control record, such as Subject 11 in Table 1 where there was very little synchronous

(alpha) activity following fatigue, perhaps indicated that these people maintained a state of attentiveness approximating that before fatigue.

The period of recuperation following physical fatigue is relatively short, at least in terms of the EEG, so that 30–40 minutes after exercise the EEG is back to its original state, indicating that the individual has returned to his previous state of attentiveness and relative metabolic balance. In previous reports in the literature on EEG changes in fatigue, some forms of mental activity were contributing factors in the state of fatigue.

## *Summary*

1. EEG studies on 16 human subjects were performed in the rested state and immediately following strenuous physical exercise, and the changes in amplitude and per cent time alpha (alpha index) were noted.

2. There was a significant and consistent increase in the alpha index of all subjects following exercise, and in the appearance of alpha rhythm in some subjects in which alpha had been absent prior to the exercise. Subjects with moderate alpha activity prior to physical fatigue demonstrated more dramatically the increase in alpha index.

3. In those records showing an increase greater than 5% above the control record, the average increase in alpha following physical fatigue was 22–28% greater in the frontal to central bipolar leads, and 17–19% more alpha in the central to occipital bipolar leads.

4. The average increase in amplitude ranged from 15 microvolts in the right occipital region to 19 microvolts in the right frontal region, and an average of 16 microvolts increase on the left following physical fatigue.

5. The various factors involved which might have produced the EEG changes observed after physical fatigue are discussed.

## *Acknowledgments*

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# A CYTOCHEMICAL TEST FOR DETECTION OF ANTIBIOTICS EFFECTIVE AGAINST PSITTACOSIS VIRUS[ad]

MORRIS POLLARD, RICHARD W. MOORE, YOH TANAMI[b] AND T. J. STARR[c]

Investigations on psittacosis virus in human cells have revealed a unique cytochemical sequence which was associated with replication of the virus (16, 21). Human cells, infected with psittacosis virus, were stained with acridine orange and examined by ultraviolet microscopy. Under such circumstances, cytoplasm and nucleolus (representing RNA material) stained red, and nucleus (representing DNA material) stained yellow-green (1). Infectious psittacosis virus particles complexed with acridine orange and were stained yellow-green (DNA). Examinations of tissue cultures at intervals following addition of virus to the cells revealed a well defined chemical sequence by which viral replication occurred: (A) the DNA-staining virus entered the cytoplasm and was surrounded by a dense red-staining (RNA) matrix; (B) the RNA-staining sphere ("red-ball") enlarged, increased in number and started to change color; (C) as the RNA-staining material changed color through a sequence of red, orange and yellow, the yellow-green stained mature virus appeared. At the latter stage, the virus inclusion adsorbed fluorescein-tagged antibody, thus indicating serological maturity. Only serologically mature virus was infectious. According to the strain of virus inoculated, the replication cycle occurred in from 36 to 72 hours. Mature virus eventually filled the cytoplasm, the cell ruptured and each liberated virus particle was able to infect a new cell.

The cytochemical sequence shown by replicating psittacosis

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[d] Supported by funds from USPH Grant E2983.

virus is dynamic and definitive. Fluorinated pyrimidines (12) were incorporated into this virochemical process and the reaction was visualized as amassed noninfectious, abnormal ("fraudulent") viral RNA (17) or as incomplete viral DNA (18) in the cytoplasm of infected cells. The fluorochrome procedure, therefore, served to demonstrate a reaction which had been detected earlier by conventional chemical procedures (11).

The effects of antibiotics on the cytochemical sequence have been noted, thus affirming earlier predictions that this phenomenon would provide a chemical test for screening therapeutic drugs for psittacosis virus (19). In this technique, the virus-inhibitory drugs interfered with the visible cytochemical sequence along which replicating virus usually matured.

## *Methods*

The techniques of tissue propagation, virus inoculation and examinations have been described; (21) therefore, only a brief resume will be given here.

*Tissue Cells.* A line of human cells, derived from synovial tissue and designated "McCoy" (8), was used in all of the studies reported here. The McCoy cell was highly susceptible to psittacosis virus (23). The cells were grown on cover slips in Leighton tubes with medium consisting of Mixture 199 with 0.5% LAH, 2% heat-inactivated calf serum and 0.1 mg. streptomycin per ml medium. Cell cultures were washed twice with Mixture 199 and then inoculated with sufficient virus (0.1 ml) to infect about 90% of the cells. One ml of nutrient medium was then added to each tube, and cultures were thereafter incubated at 37° C. At virus inoculation time, or at intervals thereafter, nutrient medium with graded doses of one antibiotic agent was added to groups of cell cultures. The cell cultures were examined subsequently by fluorochrome microscopy (21). Duplicate cultures were assayed for virus content by replacing the nutrient fluid with antibiotic-free medium, freezing twice to lyse the cells, and inoculation of this material into mice intracerebrally and into fresh tissue cultures for particle count determinations (23).

*Antibiotics.* Antibiotics were commercially available drugs. They were stored as concentrated solutions in distilled water at −20° C. until used. Graded doses (100 ug-0.75 ug/ml) of each drug was added to groups of McCoy cell cultures at intervals following addition of virus to the cells. The drugs used were dihydrostreptomycin sulfate, penicillin, kanamycin, erythromycin, chloromycetin, tetracycline, oxytetracycline and chlortetracycline HCL (CTC). In addition, tylosin tartrate* and several derivatives thereof were studied for antiviral effect.

*Virus.* The psittacosis virus was originally recovered from turkeys by Boney *et al.* (4), and has since then been propagated in allantoic fluid through

* Eli Lilly and Company, Indianapolis, Indiana.

numerous chick embryo passages. This strain is toxigenic; it reproduces very rapidly and has been designated "malignant" in contrast to less-invasive, "benign" strains which have been described. The virus was stored in allantoic fluid at −50° C. until used.

*Microscopy.* Each cell culture was fixed in ethanol-acetic acid (2:1), washed in buffer (pH 3.8), stained in 0.01% acridine orange in buffer, washed and mounted in the buffer. The cultures were observed with a Reichert Zetopan ultraviolet microscope and microphotographs were made on Kodak High Speed Type B Ektachrome film.

## *Results*

The DNA of psittacosis virus entered the cytoplasm and was surrounded by a cell-contributed matrix of RNA. At 14 hours after inoculation this gave the appearance of a dense red sphere, distinct from the cytoplasm, thus, the basis for the designation of "red ball" stage (Fig. 1). The "red balls" increased in size and number and as each approached maturity it went through a color sequence of red, orange, yellow to green (Fig. 2). At the latter stage each mass subdivided into pinpoint virus particles which, as serologically and biologically mature virus, adsorbed fluorescein-tagged antibody selectively.

Large doses of streptomycin had neither microscopic nor biological effect on psittacosis virus. All of the remaining drugs inactivated extracellular virus; however, within the designated list of drugs a distinct spectrum of activity on intracellular virus was shown. Treatment of virus at the "red ball" stage with CTC or tylosin tartrate (in doses as low as 1 ug per ml nutrient medium) resulted in sudden interruption of virus maturation (Fig. 1). Virus in untreated control cultures proceeded to maturity (Fig. 2). A drug dose-time relationship was demonstrated: If the drug was removed early, viral activity was resumed. The remaining drugs showed a gradient of decreasing activity against intracellularly established virus, as follows: Oxytetracycline, tetracycline, chloromycetin, erythromycin, kanamycin and penicillin. Only two drugs (CTC and tylosin) showed microscopically detected anti-viral activity and this was confirmed by assay of treated cultures for infectivity (Table 1).

The treatment of intracellular mature virus with CTC and tylosin interrupted continued replication of virus; however, the cytochemical display was not as distinct as with treatment at earlier stages of virus replication.

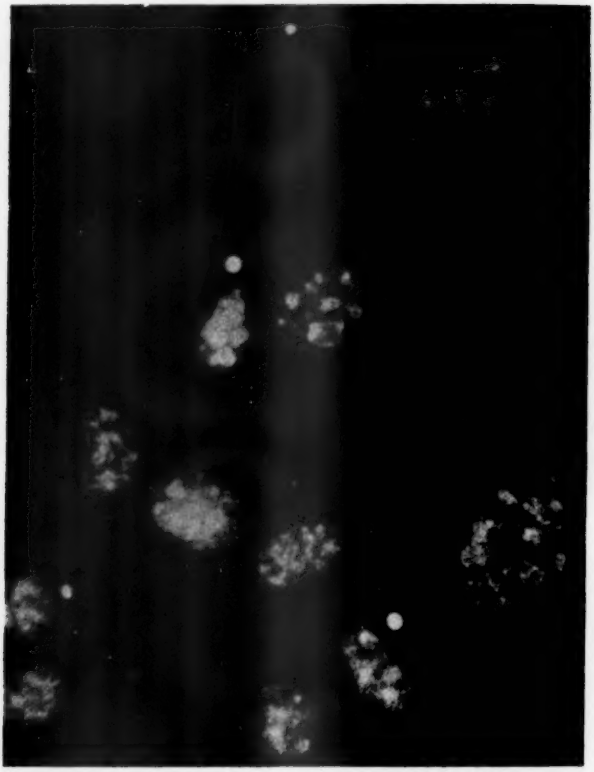

FIG. 1. Black and white print from an Ektachrome transparency. The white sphere appears red in the original slide and represents the "red ball" stage of replicating psittacosis virus in McCoy cell. Acridine orange, ultra violet light X1000.

A number of tylosin derivatives were tested on cells showing the "red ball" stage of psittacosis virus. As shown in Table 2, the tartrate and gluconate derivatives were most active; the lactate and acetyltartrate derivatives less so and the sulfate and desmycosin forms were cytotoxic and inactive.

## *Discussion*

Psittacosis virus represents a taxonomic bridge between rickettsiae and the large viruses. It shows a relationship to the

former in responding to treatment with tetracyclines, and to the latter in replicating through a stage in which it is not infectious. Agents which interfere with the cytochemical pattern of replication have potential therapeutic value; and beyond this, the procedure should serve as a tool for the study of biochemical phenomena unrelated to the disease which this virus causes.

The therapeutic value of an antiviral drug may be predicated on its ability to penetrate the infected cell; those which are re-

FIG. 2. Black and white print from an Ektachrome transparency. The masses of psittacosis virus in the cytoplasm of the McCoy cell are green-yellow in the color preparation and represent mature particulate virus. Acridine orange, ultra violet light, X1000.

TABLE 1

*Effects of antibiotics on replication of psittacosis virus in McCoy cells*

| Drug | ug/ml | Microscopic examination | Virus recovery 4 days after treatment | |
|---|---|---|---|---|
| | | | Mouse inoculations* | Particles/ml† |
| Tylosin tartrate | 25 | Inhibited | 0/6 | 0 |
| Tylosin tartrate | 0.75 | Inhibited | 0/6 | 0 |
| Chlortetracycline HCL | 25 | Inhibited | 0/6 | 0 |
| Chlortetracycline HCL | 1.5 | Inhibited | 0/6 | 0 |
| Streptomycin | 200.0 | Maturation | 6/6 | $2 \times 10^5$ |
| Control | .... | Maturation | 6/6 | $2 \times 10^6$ |

* Mice infected/mice inoculated.
† As determined in tissue culture.

TABLE 2

*Inhibitory effect of Tylosin and derivatives on initial stage of psittacosis virus in McCoy cells*

| | Tylosin preparation | Code | Effect of dosage (ug.) per ml nutrient medium | | |
|---|---|---|---|---|---|
| | | | 25 | 12 | 6 |
| I. | Tartrate | 314–169AD–283 | Inhibited* | Inhibited* | Inhibited* |
| II. | Tartrate | 238–134–063 | Inhibited* | Inhibited* | Inhibited* |
| III. | Tartrate | 381–155AD–284 | Inhibited† | Inhibited† | Inhibited† |
| IV. | Tartrate | 361–322B–178–1 | Inhibited† Cytotoxic | Inhibited† | Inhibited† |
| V. | Base | 316–285AD–66 | Insoluble | | |
| VI. | Gluconate | 361–322B–90–2 | Inhibited* Sl. Cytotoxic | Inhibited* | Inhibited* |
| VII. | Acetyl d-tartrate | 238–169–001 | Inhibited¶ Cytotoxic | Inhibited¶ | Inhibited¶ |
| VIII. | Lactate | 294–172AD–264A | Inhibited¶ Cytotoxic | Inhibited¶ Sl. Cytotoxic | Inhibited¶ |
| IX. | Sulfate | 361–322B–181–2 | Inhibited§ Cytotoxic | Inhibited§ | Not inhibited‖ |
| X. | Desmycosin | 361–4700–126–6 | Inhibited* Cytotoxic | Not inhibited‖ Sl. Cytotoxic | Not inhibited‖ |

* Complete inhibition.
† Some increase in number of viral foci, no maturation.
¶ Marked increase in number of viral foci, no maturation.
§ Suggestive maturation.
‖ No inhibition, maturation of virus.

stricted to the exterior of the cell would have little effect on intracellular virus. CTC and tylosin tartrate appear to interfere with the reaction triggered by viral DNA in the formation of its surrounding RNA matrix. This suggested that an enzyme block has occurred but the mechanism of this reaction has not yet been determined. The reversing effect of riboflavin on aureomycin inhibition, as reported by Foster and Pitillo (13) with a bacterial system, could not be demonstrated with psittacosis virus. The virus-inhibiting effect of aureomycin contrasts with the accumulation of abnormal RNA and DNA following treatment of infected cells with fluorinated pyrimidines (17) and indicates that different mechanisms prevail. Tetracycline drugs have shown therapeutic value in animals infected with psittacosis and related agents (6). CTC cleared psittacosis-infected birds when it was administered in an oily suspension as an intramuscular depot (5) and in feed (7, 14). The effects of other drugs on such infections were equivocal.

Tylosin is a macrolide-type antibiotic which has shown a destructive effect on PPLO and on some bacterial agents (10, 13). It has been used principally in the treatment of diseased poultry (2) and as a feed supplement for swine. Its effect on psittacosis virus was anticipated on the basis of a test on mice infected with meningopneumonitis virus. Trachoma virus replicated in human cells through a cytochemical sequence similar to that described for psittacosis virus (3, 20). Tylosin tartrate and CTC interrupted the virus maturation sequence (22) following the addition of each drug to the nutrient fluid of trachoma-infected cultures.

An obvious benefit of the cytochemical procedure described here is in the accurate representation of virus activity by its appearance with fluorochrome microscopy. The accuracy of interpretation with this technique has been documented here with antibiotics and elsewhere with fluorinated pyrimidines (17), folic acid antagonists (15) and amino acid analogues (22). Such an approach to the study of antibiotic effect on a related human pathogen (i.e., trachoma) may obviate the necessity of performing virus assays in human volunteers. Indeed, this procedure is is now being applied to trachoma infection with encouraging results.

*Summary*

The replication of psittacosis virus in cells was accompanied by a sequence of cytochemical changes which reflected nucleic acid metabolism. Drugs with therapeutic effect on this virus interfered with biosynthesis of its nucleic acid and this was observed as an interruption in the sequence of cytochemical changes. On this basis, tylosin tartrate, in addition to chlortetracycline hydrochloride, were therapeutically useful in psittacosis infection.

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# ANTITUMOR AND TOXICITY PROPERTIES OF SOME CARBODIIMIDES

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The carbodiimides appear to be a class of compounds which have not been tested for their antitumor properties. Their chemistry has been reviewed (3). In earlier literature they were named as derivatives of cyanamide. They may be visualized as substituted ureas with one molecule of water removed.

$$R-\underset{H}{N}-\overset{O}{C}-\underset{H}{N}-R \qquad R-N=C=N-R+H_2O$$

In the course of our cancer chemotherapy program, one of these, the N,N′-dicyclohexylcarbodiimide (DCC), was found to have some activity against Ca-755 at 10 mg/kg in mice. Therefore, several other carbodiimides were evaluated for antitumor activity and all were tested for toxicity to mice, bacteria, yeast and tissue cultures.

## *Materials and Methods*

Four carbodiimides and one urea analogue were used in this investigation. They were the DCC, N,N′-diisopropylcarbodiimide, N,N′*p*-tolylcarbodiimide, and bis(triphenylmethyl)carbodiimide, all from Aldrich Chemical Company, Milwaukee, Wisconsin, and N,N′-dicyclohexylurea, a by-product of peptide synthesis in this laboratory.

The antitumor activities of these compounds were determined as described in *Cancer Chemotherapy Reports* (1). For the acute and chronic toxicity tests in mice, the compounds were ground into 1.0% carboxymethylcellulose solution of medium viscosity. Serial dilutions in saline were prepared in multiples of ten. Intraperitoneal injections were given daily except Sunday. The observation periods for acute and chronic toxicities were seven and 30 days, respectively. The dose for the acute toxicity was ten times greater than the highest dose used in the chronic toxicity test, except for the bis(triphenyl)-methyl derivative which was five times greater. At the end of the observation periods the surviving mice were autopsied and the gross pathology was observed.

The toxicities of these compounds were also tested on seven bacterial species

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and one yeast.[b] The basal medium used with *S. faecalis, L. casei, L. citrovorum,* and *L. plantarum* has been described in previous work from this laboratory (5). *E. coli* was grown in Davis broth (2). *S. aureus, Ps. aeruginosa,* and *S. carlsbergensis* were grown in Difco dextrose broth. The test compounds were added aseptically to the autoclaved media. The results were determined after an 18-hour growth period, for reasons previously described (5).

The toxic effects of these compounds were further tested on the KB cell line and the Fernandes strain of amnion according to the CCNSC specifications for screening in tissue culture (1). The reported results are average values obtained from three screening experiments for each compound.

## *Results*

Tumor-to-control values (T/C)* obtained by the CCNSC and by us are give in Table 1. The CCNSC tested the DCC four times at the 10 mg/kg level against Ca-755 in mice. T/C values of .30, .59, and .49 were reported for three tests, with excessive toxicity in one trial. The cumulative T/C value was .08. The DCC was inactive for S-180 and Le-1210. The other carbodiimides were

TABLE 1

*Antitumor activities of some carbodiimides against four mouse tumors**

| Compound | Dose mg/kg | T/C† values for Ca-755 | Sa-180 | Ehrlich ascites | Le-1210 |
|---|---|---|---|---|---|
| DCC | 62.5 | | .91‡ | | |
| DCC | 10. | .46‡¶ | .88 | | 1.18‡ |
| DCC | 5. | | | .71 | |
| Diisopropyl | 10. | | .83 | 1.80 | |
| Ditolyl | 15. | | .92 | .73 | |
| Bis(triphenyl)methyl | 30. | | .96 | 1.20 | |
| Bis(triphenyl)methyl | 450. | .95‡ | .78‡ | | .98‡ |
| Dicyclohexylurea | 450. | 1.13‡ | | | .88‡ |
| Dicyclohexylurea | 500. | | .78‡§ | | |

* The diallyl derivative has been reported as non-effective in mouse tumors (4).
† The T/C value of a compound is the figure one obtains by dividing the weight of the tumor in treated animals (in mgm.) by the weight of the tumor in control animals (in mgm.).
‡ Results from CCNSC.
¶ Average of three tests.
§ Average of two tests.

[b] The authors gratefully acknowledge the capable assistance of Vivian Gold, whose work was supported by Grant Cy-3609 of the CCNSC.

* The T/C value of a compound is the figure one obtains by dividing the weight of the tumor in treated animals (in mgms.) by the weight of the tumor in control animals (in mgms.).

inactive toward S-180 and Ehrlich ascites at 15 and 30 mg/kg.

The acute toxicity of the DCC in mice was over 100 mg/kg, whereas the chronic toxicity range was more than 1 and less than 10 mg/kg (Table 2). The reverse was true of the diisopropyl

TABLE 2

*Toxicity of the carbodiimides in mice*

| | Chronic toxicity (30-day observation) | | | | |
|---|---|---|---|---|---|
| | DCC | Diisopropyl | Ditolyl | Bis-(triphenyl)-methyl | Dicyclo-hexyl-urea |
| Dose mg/kg* | Day of death‡ | Day of death | Day of death | Day of death | Day of death |
| .1 | 23 | 24 | | 23,29 | |
| .3 | | | 12,20,24,30 | | |
| 1. | . . | 6 | | 24 | |
| 3. | | | 7,12,14,21,21 | | |
| 10. | 13,14,20,20,20 | 9 | | 29 | |
| 30. | | | 6,6,9,11,12,13 | | |
| 100. | | | | . . | |
| | Acute toxicity (7-day observation) | | | | |
| 100. | 6 | 2,2,3,4,4 | | . . | |
| 300. | | | 1,1,1,1,1,2 | | |
| 500. | | | | . . | 3,7 |

* Six mice injected on each dilution (doses from .01–.001 were ineffective.
‡ Each figure in Day-of-Death columns represents one mouse dead at × number of days after initial injection.

derivative, in which the acute toxicity was high and the chronic toxicity low. The ditolyl derivative was very toxic in the acute test. In the chronic test the toxic effect seemed to be cumulative, with over half of the mice dying at the 0.3 mg/kg level. The bis(triphenyl)methyl derivative was not toxic in any dilution used.

At the end of the chronic and acute toxicity tests, the surviving mice were autopsied for gross pathological effects. No abnormal conditions could be detected in mice surviving DCC or diisopropylcarbodiimide treatment. During the test, the mice treated with the ditolyl derivative demonstrated hemorrhagic and flaccid intestines immediately below the stomach. On autopsy, the adrenals of these animals were red, but other organs appeared normal. Dosages of 1–100 mg/kg of bis(triphenyl)methylcarbodiimide caused a mottled appearance of the spleen and liver which seemed to be the result of peritonitis.

Toxicity tests were performed on eight microbial systems. The results with four of these systems are shown in Table 3. The carbodiimides showed the widest range of toxicity to the yeast, *s. carlsbergensis*. The $ID_{50}$ with the ditolyl derivative was <20 μg/ml, and 500 for the bis(triphenyl)methyl derivative. *S. aureus* showed the same range of toxicity, but did not appear to have the selectivity of *s. carlsbergensis*. *S. aureus* showed more specificity than the *L. citrovorum* and *L. plantarum*. The diisopropyl and bis(triphenyl)methyl derivatives were relatively non-toxic (>500 μg/ml) to the last two species of bacteria. *S. faecalis*, *L. casei*, *Ps. auruginosa* and *E. coli* had no value as screening agents for the compounds tested.

TABLE 3

*Toxicity of the carbodiimides in microbial systems*

| Compound | $ID_{50}$ (μg/ml)* | | | |
|---|---|---|---|---|
| | *S. carlsbergensis* | *Staph. aureus* | *L. citrovorum* | *L. plantarum* |
| DCC | 50. | 100. | 125. | 125. |
| Diisopropyl | 100. | 500. | 500. | 500. |
| Ditolyl | < 20. | 20. | 50. | 20. |
| Bis(triphenyl)methyl | 500. | 500. | >500. | >500. |
| Dicyclohexylurea | 600. | 500. | 500. | 500. |

* 18-hour readings.

The effect of the four carbodiimides and dicyclohexylurea on cells in tissue culture are shown in Table 4. The KB (malignant) cells were generally more sensitive to the carbodiimides than the amnion cells, which were of nonmalignant origin. The ditolyl and bis(triphenyl)methyl derivatives were relatively non-toxic ($ID_{50}$ = >100 μg/ml) to amnion cells, while the KB cells had an $ID_{50}$ of 4.0 μg/ml and 0.4 μg/ml, respectively. Both cell types were sensitive to diisopropylcarbodiimide ($ID_{50}$ = about 4 μg/ml). The DCC was also rather toxic to both the KB ($ID_{50}$ = 0.3 μg/ml) and the amnion ($ID_{50}$ = 10.0 μg/ml) cell strains. Its urea analogue was relatively non-toxic to both cell types ($ID_{50}$ = >100 μg/ml).

## *Discussion*

The toxicity of the carbodiimides for bacteria, mice, and human cells in tissue culture, and the weak antitumor activity of DCC are in sharp contrast to the innocuous behavior of the dicyclohexylurea. The biologic properties of the compounds may be attributed, therefore, to the carbodiimide moiety. However, variation of the substituent groups does affect toxicity significantly, and in a different manner in each group. These data suggest that with appropriate substitution, significant antitumor activity may possibly be found associated with limited toxicity. This view is supported by the lesser toxicity of some carbodiimides for the amnion cell as compared to the KB cell.

## *Summary*

The comparative toxicities of four carbodiimides have been determined in mice, seven bacteria, one yeast, and two human cell lines.

The bis(triphenyl)methyl derivative showed a high degree of specificity between normal and malignant cell lines. The dicyclohexyl derivative (DCC) also showed specificity in tissue culture. It was the only one to show antitumor activity in mice.

TABLE 4

*Toxicity of the carbodiimides in tissue culture systems*

| Compound | $ID_{50}$ (μg/ml) | |
|---|---|---|
| | KB | Amnion |
| DCC | 0.3 | 10.0 |
| Diisopropyl | 4.0 | 4.5 |
| Ditolyl | 4.0 | >100.0 |
| Bis(triphenyl)methyl | 0.4 | >100.0 |
| Dicyclohexylurea | >100.0 | >100.0 |

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# PEPTIDE STUDIES IV: INHIBITION OF BACTERIAL GROWTH BY DI- AND TRIPEPTIDES

S. SHANKMAN, V. GOLD AND S. HIGA[b]

Earlier, (12) L-valyl-L-valyl-D-valine was found to be the only antibacterial tripeptide of valine of the 5 valine tripeptides evaluated. Extension of this work indicated that the dipeptide L-valyl-L-valine could be inhibitory under selected conditions. Since synthesis of many tripeptides for comparison of structure to antibacterial potency is a laborious task, it seemed desirable to investigate the antibacterial effect of other dipeptides and its relation to structure in an effort to postulate a limited number of tripeptides with enhanced antibacterial activity. Evidence of relation of structure to antibacterial activity constitutes the present report, together with data on reversal of the activity of the L-valyl-L-valyl-D-valine.[c]

*Experimental*

The main organisms employed were *Lactobacillus plantarum* (A.T.C.C. #8014), formerly named *Lactobacillus arabinosus; Pediococcus cerevisiae* Cal. (A.T.C.C. #8081), obtained from the University of California in 1953; and *Pediococcus cerevisiae* (A.T.C.C. #8081), obtained from the American Type Culture Collection in 1956. The *P. cerevisiae* was previously named *Leuconostoc citrovorum*. The differences in the two cultures has been described (13). They are designated here as ATCC and Cal. *L. plantarum* was maintained as stabs on yeast-dextrose agar. Both cultures of *P. cerevisiae* were kept on slants containing 2 gm. each of yeast extract, peptone and glucose, 0.2 gm. of $KH_2PO_4$ and $MgSO_4.7H_2O$, 3 gm. $CaCO_3$, and 4 gm. agar per 200 ml. A peptone-glucose-yeast extract medium (13) was used for liquid culture for inocula for all of the lactic cultures. *Lactobacillus casei* (A.T.C.C. #7469) and *Leuconostoc mesenteroides* (A.T.C.C. #8042) were maintained on yeast-dextrose agar. *Lactobacillus fermenti* (A.T.C.C. #9338) was kept on tomato juice agar (Difco). *Streptococcus faecalis* (A.T.C.C. #8043) was maintained according to the A.O.A.C. (1) *Escherichia coli* W, wild strain, and the valine-requiring *E. coli* mutant #48–62 (4) were tested on the

[a] Supported in part by Grant CY-3609 from the National Institutes of Health.

[b] Pasadena Foundation for Medical Research, Pasadena, California.

[c] Abbreviated *LLD*.

medium of Davis (5) supplemented with 10 μg/ml of L-valine. They were maintained on the same medium supplemented with yeast extract and casein hydrolysate.

Complete amino acid mixtures (13) were used as test media for all organisms except *E. coli*, since some peptides are resistant to acid hydrolysis (3, 14) and may occur in casein hydrolysate. The media were modified as follows: For *P. cerevisiae* ATCC, one third the total amino acids; for *P. cerevisiae* strain Cal., 37 mgm additional histidine (15) per liter of single strength medium (13). One drop of dilute washed inocula, standardized to an O.D. of .02, was used per 4 ml. tube. Response was measured after 16–20 hours incubation with a Beckman colorimeter, model C, with a 600 mμ filter. The highest level of inhibitor was 0.5 mg/ml. final volume. Test compounds were autoclaved with the medium. The valine peptides and other derivatives were made as previously described (14). Commercial sources were used for other peptides with exceptions as noted.

## *Results*

Data are given as $ID_{50}$, the concentration of inhibitor needed for half maximum inhibition (7). Prolonged growth periods, heavy inocula, or basal media which promote early, heavy growth all tended to obscure the inhibitory effects of peptides (as shown in Figures 1, 2). The *LLD* was employed as a positive control in each experiment.

Data presented in Tables I-IV establish that several L-L dipeptides inhibit bacterial growth with efficacy, dependent on their amino acid composition. Glycyl peptides of serine, threonine, tyrosine, histidine, proline, asparagine, glycine, leucine and valine inhibit weakly, if at all. The glycyl peptides of methionine, phenylalanine and tryptophane inhibit *P. cerevisiae* Cal. at a concentration for half-maximum growth ($ID_{50}$) of .03 mg/ml or less. Glycyl-tryptophane (Table I) and leucyl-tryptophane inhibited *L. plantarum* ($ID_{50}$ of .01 and .02 respectively), *P. cerevisiae* ATCC (.01 and .01) and *P. cerevisiae* Cal (.03 and .01). On varying the N-terminal amino acid (Table III), maximal inhibition was found with arginyl-leucine and decreased in the order of arginine > leucine ⩾ valine > glycine > alanine > β-alanine. These peptides did not inhibit *L. plantarum*.

Inhibition for both strains of *P. cerevisiae* vanished in the di- and tripeptides of leucine, glycine, tyrosine, and asparagine (listed in Table II) when a D amino acid was substituted for the L amino acid in either the C- or N-terminal position of a dipeptide or the N-terminal of a tripeptide. The L-leucyl peptides of L- and

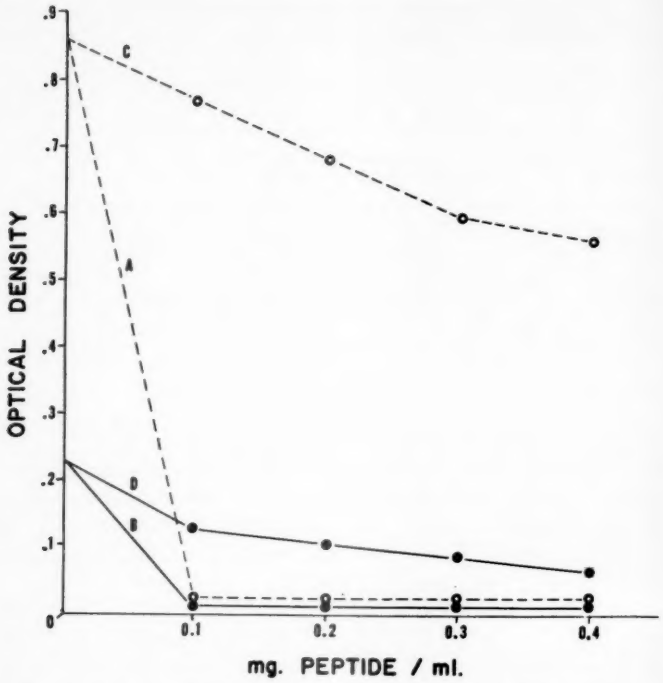


FIG. 1. Response of *P. cerevisiae* ATCC to *LLD* and L-valyl-Lvaline.
Curve A LLD 1–1,000 inoculum
Curve B LLD 1–10,000 inoculum
Curve C LL 1–1,000 inoculum
Curve D LL 1–10,000 inoculum

D-alanine (L- and D-alanine are both utilized by *P. cerevisiae* (11)) did not follow this general pattern. In the valine tripeptides, the N-terminal D-containing peptide of the configuration D-L-L did not inhibit significantly whereas the C-terminal D-containing L-L-D did inhibit.

Symmetrical peptides (Table IV) are frequently more inhibitory than asymmetrical. Maximum inhibition was observed for phenylalanyl-phenylalanine and methionyl-methionine.

Of the 8 tripeptides of L- and D-valine, only *LLD* inhibited the 18 hour growth of the 3 organisms completely under these conditions. The concentration of *LLD* for half maximal growth ($ID_{50}$)

averaged .03 mg/ml in many trials. The value was about the same for *L. plantarum* and for *P. cerevisiae* ATCC and Cal. The dipeptide L-valyl-L-valine inhibited the growth of both *P. cerevisiae* with an $ID_{50}$ of .5 for the ATCC strain and of .1 to .6 for

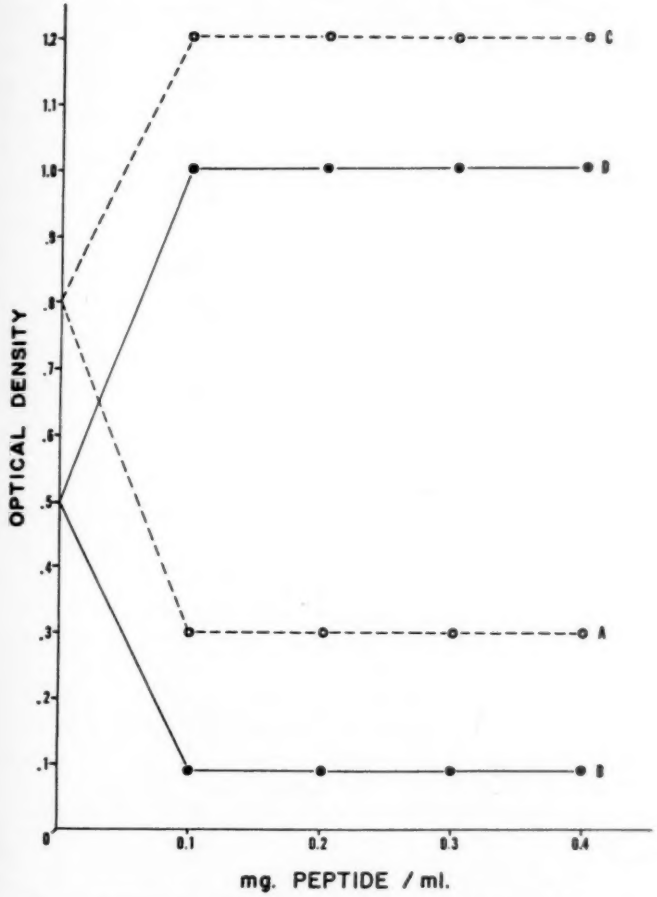


FIG. 2. Response of *L. plantarum* to *LLD* and L-valyl-Lvaline.
Curve A LLD 1–2,000 inoculum
Curve B LLD 1–10,000 inoculum
Curve C LL 1–2,000 inoculum
Curve D LL 1–10,000 inoculum

TABLE 1

*Effect of C-terminal amino acid on $ID_{50}$ of dipeptides*

| Compound | $ID_{50}$ mg/ml P. cerevisiae | |
|---|---|---|
| | ATCC | Cal. |
| glycyl-DL-α-amino-butyric acid | N† | I† |
| glycyl-L-asparagine | I | .2 |
| glycyl-DL-ethionine | .002‡ | .003 |
| glycyl-L-glutamic acid | .1 | .1 |
| glycyl-glycine | N | I |
| glycyl-L-histidine . HCl | N | N |
| glycyl-L-leucine | N | .2 |
| glycyl-DL-methionine | N | .05 |
| glycyl-DL-norleucine | .01‡ | .02 |
| glycyl-DL-norvaline | N | I |
| glycyl-DL-phenylalanine | I,N | .06 |
| glycyl-L-proline | N | N |
| glycyl-DL-serine | N | I |
| glycyl-L-threonine . $2H_2O$ | N | I,N |
| glycyl-L-tryptophane | .01‡ | .03 |
| glycyl-L-tyrosine | N | .4 |
| glycyl-L-valine | N | I |
| DL-ethionine | .3 | I‡ |
| DL-norleucine | I | I‡ |
| DL-tryptophane | N | N‡ |

† N = no inhibitory effect, I = less than 50% inhibition at 0.5 mg/ml.
‡ These data also obtained with *L. plantarum*. In Tables 1–4, other compounds did not inhibit *L. plantarum*.

TABLE 2

*Effect of optical isomerism on $ID_{50}$ of dipeptides*

| Compound | $ID_{50}$ mg/ml P. cerevisiae | |
|---|---|---|
| | ATCC | Cal. |
| L-leucyl-glycine | N | I |
| D-leucyl-glycine | N | N |
| L-leucyl-glycyl-glycine | I | I |
| D-leucyl-glycyl-glycine | N | S† |
| L-leucyl-L-tyrosine | .03 | .01 |
| D-leucyl-L-tyrosine | N | N |
| glycyl-L-asparagine | I | .2 |
| glycyl-D-asparagine | N | N |
| L-leucyl-L-alanine | N | .5 |
| L-leucyl-D-alanine | I | .2 |

† S = stimulation. N, I as in Table 1.

TABLE 3

*Effect of N-terminal amino acid on $ID_{50}$ of dipeptides*

| Compound | $ID_{50}$ mg/ml P. cerevisiae | |
|---|---|---|
| | ATCC | Cal. |
| L-alanyl-L-leucine | N | I |
| L-arginyl-L-leucine · acetate | .02 | .005 |
| glycyl-L-leucine | I | .2 |
| L-leucyl-L-leucine | .1 | .3 |
| L-prolyl-L-leucine | N | I |
| L-valyl-L-leucine | N | I |
| glycyl-L-tyrosine | N | .4 |
| leucyl-L-tyrosine | .02 | .01 |
| valyl-L-tyrosine | .02 | .03 |
| L-alanyl-L-asparagine | N | .4 |
| glycyl-L-asparagine | I | .2 |
| L-leucyl-L-asparagine | .2 | .02 |
| DL-alanyl-DL-phenylalanine | .2 | .1 |
| β-alanyl-DL-phenylalanine | I | .4 |
| glycyl-DL-phenylalanine | I | .03 |
| L-leucyl-L-phenylalanine | .06 | .06 |
| L-phenylalanyl-L-phenylalanine | <.01 | <.01 |
| DL-valyl-DL-phenylalanine | .1 | .05 |
| DL-alanyl-glycine | N | N |
| cystinyl-glycine | .2 | N |
| L-leucyl-glycine | N | I |
| L-phenylalanyl-glycine | I | .08 |
| L-prolyl-glycine | N | I |
| DL-valyl-glycine | N | N |

TABLE 4

*$ID_{50}$ of some symmetrical peptides and dipeptide anhydrides*

| Peptide | $ID_{50}$ mg/ml P. cerevisiae | |
|---|---|---|
| | ATCC | Cal. |
| DL-histidyl-DL-histidine | .5 | N |
| L-valyl-L-valine | .5 | .1 to .6 |
| L-leucyl-L-leucine | .1 | .3 |
| L-phenylalanyl-L-phenylalanine | <.01 | <.01 |
| L-methionyl-L-methionine | <.01 | .02 |
| phenylalanyl-lysyl anhydride | N† | N† |
| valyl-lysyl anhydride | N† | N† |

† Each of the 4 optical isomers (L-L, L-D, D-L, D-D) was tested separately.

Cal. strain. The valyl-valines L-D, D-L and D-D did not inhibit. The valyl- or phenylalanyl-lysyl anhydrides also did not inhibit. Some L-L-L tripeptides (Table V) were weak inhibitors.

TABLE 5

*$ID_{50}$ of tri- and tetrapeptides*

| Peptide | $ID_{50}$ mg/ml *P. cerevisiae* | |
|---|---|---|
| | ATCC | Cal. |
| glycyl-glycyl-glycine | N† | N |
| L-valyl-L-valyl-L-valine | N† | I |
| L-valyl-L-valyl-D-valine | .03† | .02 |
| L-valyl-D-valyl-L-valine | N† | N |
| L-alanyl-L-alanyl-D-alanine | N† | N |
| L-alanyl-D-alanyl-L-alanine | N† | N |
| glycyl-L-alanyl-D-alanine | N† | N |
| glycyl-L-leucyl-glycine | N† | I |
| glycyl-L-leucyl-L-leucine | I | .5 |
| glycyl-L-leucyl-L-tyrosine | .1 | .3 |
| gly.gly.gly.gly | N† | I |
| ala.ala.ala.ala (LDLL) | N† | .3 |

† These data were also obtained with *L. plantarum*.

*L. plantarum* was not inhibited at 72 hours by the L-L-D trivaline on a medium lacking pyridoxine, pyridoxal and pyridoxamine, although less total growth occurred on this medium. *S. faecalis*, *L. mesenteroides*, *L. fermenti*, *E. coli* strain W, and *E.* coli 48–62 were not inhibited by the L-L-D trivaline under a variety of conditions. *L. casei*, an organism with pronounced hydrolytic properties, was not inhibited by the peptides indicated in Tables I-IV, glycyl-asparagine excepted.

In 72-hour growth periods the L-valyl-L-valine was well utilized as a valine source (13), as was the L-L-L tripeptide, and to a much lesser extent the L-L-D tripeptide. These organisms did not utilize 5 of the remaining 6 tripeptides or 3 dipeptides of L- and D-valine (13).

## *Discussion*

Ethionine and norleucine are both known to be methionine antagonists (8, 9). In the present investigation, this antagonism is weak (Table 1), since methionine is present in the medium.

However, their glycyl peptides (Table 1) are potent inhibitors of the three organisms, adding to the evidence (6, 10, 2), that amino acids and peptides have different metabolic paths. The inhibition by glycyl-tryptophane (Table 1) and leucyl-tryptophane of all three cultures may by analogy be due to an as yet unknown amino acid antagonism of tryptophane magnified greatly in the peptide form. Alternatively, tryptophane peptides may be more inhibitory than other peptides, due to stability to bacterial hydrolytic attack and blockage of an essential metabolic pathway.

Whether the peptide is utilized by the organism or may inhibit it would seem to depend on the rate of hydrolysis of the peptide by the organism. Since for all peptides reported here the cleavage products are nontoxic at concentration of the inhibitor used, it may be concluded that stability to bacterial hydrolysis is necessary for antibacterial activity.

The inhibition of *P. cerevisiae* Cal. strain by the L-L-D trivaline and its competitive reversal by the L-L-L trivaline (Table 6) may be viewed in the classic anti-metabolite manner (16). That is, the *LLD* interferes with the metabolism of an unknown compound, perhaps a peptide. This interference is reversed by the

TABLE 6

*Reversal of* L-*valyl*-L-*valyl*-D-*valine inhibition of* P. cerevisiae *Cal. by* L-*valyl*-L-*valyl*-L-*valine or* L-*valyl*-L-*valine*

| Reverser mcgm/ml | Reverser LLL LLD mcgm/ml | | | | Reverser LL | | | |
|---|---|---|---|---|---|---|---|---|
| | 25 | 75 | 225 | 675 | 25 | 75 | 225 | 675 |
| 0† | .74‡ | .15 | .02 | .02 | .74 | .15 | .02 | .02 |
| .3 | .79 | .. | .. | .. | .75 | .. | .. | .. |
| 1.0 | .82 | .16 | .. | .. | .81 | .15 | .. | .. |
| 3 | .88 | .31 | .06 | .. | .78 | .18 | .02 | .. |
| 10 | .88 | .52 | .20 | .06 | .88 | .28 | .05 | .01 |
| 30 | .94 | .67 | .40 | .20 | .94 | .44 | .11 | .03 |
| 100 | .. | .73 | .54 | .39 | .. | .54 | .25 | .04 |
| 300 | .. | .. | .65 | .54 | .. | .. | .38 | .13 |
| 1000 | .. | .. | .. | .65 | .. | .. | .. | .27 |

$$\frac{\text{LLL}}{\text{LLD}} = \text{about 1}$$

† Growth without inhibitor or reverser = .95.
‡ Opt. density.

L-L-L tripeptide which has a dipeptide structure in common with the inhibitor. A schematic presentation is given in Figure 3. The ratio of *LLL* to *LLD* for reversal is about 1. L-valyl-L-valine also reverses the *LLD* noncompetitively (Table 6). However, in contrast to the competitive relationship of the tripeptides, an increase of 10 fold in the concentration of L-valyl-L-valine is required to maintain the same degree of reversal of the growth inhibition for each 3 fold increase in the amount of inhibitor. At higher concentrations, > .5 mg/ml, both *LL* and *LLL* may also inhibit *P. cerevisiae*, dependent on total growth.

For *L. plantarum* (Table 7) under the conditions of suboptimal growth necessary to observe inhibition by *LLD*, L-valyl-L-valine, L-valyl-L-valyl-L-valine, and many other all L-di and tri-peptides stimulate growth of the organism. From growth experiments only, it is not possible to distinguish between competitive reversal of the *LLD* by L-L-L configuration at a ratio of 100:1 or enhancement of growth by the L-L-L form to the level at

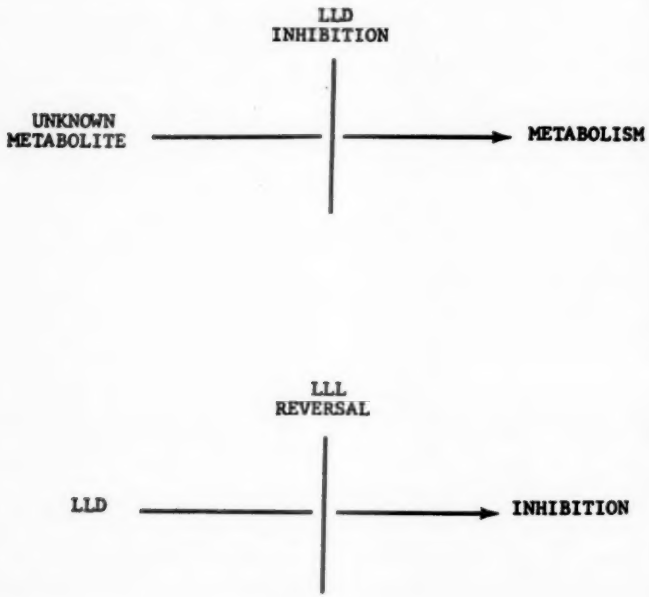


FIGURE 3

TABLE 7

*Reversal of* L-*valyl*-L-*valyl*-D-*valine inhibition of* L. plantarum *by* L-*valyl*-L-*valyl*-L-*valine or* L-*valyl*-L-*valine*

| Reverser mcgm/ml | Reverser LLL LLD mcgm/ml 25 | 75 | 225 | 675 | Reverser LL 25 | 75 | 225 | 675 |
|---|---|---|---|---|---|---|---|---|
| 0† | .08‡ | .03 | .03 | .06 | .08 | .03 | .03 | .05 |
| .03 | .10 | .. | .. | .. | .08 | .. | .. | .. |
| .1 | .13 | .07 | .. | .. | .06 | .03 | .. | .. |
| .3 | .28 | .11 | .09 | .. | .14 | .05 | .05 | .. |
| 1.0 | .46 | .32 | .25 | .22 | .10 | .05 | .08 | .10 |
| 3 | .68 | .62 | .61 | .65 | .30 | .19 | .33 | .48 |
| 10 | .. | .75 | .74 | .80 | .. | .66 | .68 | .72 |
| 30 | .. | .. | .88 | .88 | .. | .. | .78 | .79 |
| 100 | .. | .. | .. | .94 | .. | .. | .. | .85 |
| | $\frac{LLL}{LLD} = \text{about } \frac{1}{100}$ | | | | $\frac{LL}{LLD}$ not constant | | | |

† Growth without inhibitor or reverser = .34. This is not full growth.
‡ Opt. density.

which inhibitory activity is sharply diminished. The amount of either tri- or di-peptide necessary for good growth appears to be independent of the concentration of peptide inhibitor. Both peptides may be utilized in a manner bypassing the inhibited system.

From the inhibition data, minimal requirements for antibacterial activity are either an L-L dipeptide structure or an N-terminal L-L dipeptide structure in a tripeptide. The tripeptide should be free from C-terminal attack by carboxypeptidases. The L-L dipeptide should itself be maximally resistant to aminopeptidases, either by choice of naturally occurring amino acid, by insertion of a known inhibitor, or by stability conferred by the symmetrical dipeptides. Tripeptides which would incorporate these principles are the L-L-D tripeptides of phenylalanine, methionine, tryptophane, and arginine. Synthesis of these compounds to test and extend these minimal principles for antibacterial peptides is now under way.

## *Summary*

The relationship between antibacterial activity and structure of 10 tripeptides and 49 dipeptides has been investigated. Struc-

ture, composition and optical configuration of dipeptides are correlated to antibacterial activity. Principles for design of antibacterial tripeptides are proposed.

*Acknowledgment*

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# EFFECT OF RH HAPTEN INGESTION ON SERUM PHOSPHOLIPID[ab]

BARBARA TOUPIN AND BETTINA B. CARTER[c]

Incompatibility between blood types of parents is known to lead occasionally to hemolytic disease of the newborn. This is not a clinical entity, but a complex and variegated syndrome ranging from simple anemia through severe icterus to intrauterine death. The first two manifestations can be controlled largely by exsanguination transfusion procedures. The third is not amenable to this approach.

Use of a crude fraction derived from Rh positive red cells has been aimed at the prevention of intrauterine death due to Rh incompatibility. This has been given now to over a thousand women. Results from oral administration (1, 2) of the fraction, called the Rh hapten, to sensitized Rh negative women prior to a new pregnancy as well as during that pregnancy indicate success in a high percent of cases. This fraction, although not yet adequately characterized, contains phospholipids and unsaturated sterols (3).

Since this material has been used clinically and promises to be employed in the future also, it is important to know some of the effects of its ingestion on the maternal organism. One of its values as a prophylactic agent lies in the fact that the Rh hapten has no apparent ill effects on the mother or child. We are concerned here with an evaluation of the effect on blood lipid phosphorus values. A study of cholesterol blood levels following ingestion was reported previously (4).

Although a variety of workers, Bollman et al (5), McClure and Huntsinger (6), Flock and others (7), have reported increase in plasma phospholipid following fat feedings, the small quantities of the Rh hapten administered to the Rh sensitized

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woman—100 to 400 mg. daily for from 15 to 20 months—would seem to preclude our suspecting any such effect from this source. That the Rh hapten might trigger an endogenous increase is not beyond the realm of possibility, drawing deductions, as we are, from Artom's work (8).

One must take into account also that Boyd (9), Tyler and Underhill (10), Oser and Karr (11) and Peters et al (12) have reported increases in plasma phospholipid levels during pregnancy. Other provocative angles include Chino's (13) observation that the immunization of rabbits led to a rise in the phospholipid content of the immune serum and the report of Aldersberg and co-workers (14) that administration of ACTH produces a slight increase in plasma phospholipids. Any or all of these factors could apply, directly or indirectly, to the oral administration of Rh hapten: involved are lipid feedings (however small), pregnancy, high degrees of immunization and, lastly, with the Rh hapten we are able to reduce antibody titers as demonstrated in a plasma medium (15).* ACTH suppresses antibody production: is there any correlation of effect here?

### *Methods*

We chose to study serum because this is readily available to us for Rh antibody titrations; also Boyd (9) considers the lipemia of pregnancy to be limited to the plasma. Initially we made use of Fiske and SubbaRow's (16) method for determining lipid phosphorus values, but later we changed to the method of Zilversmit and Davis (17) which proved very satisfactory. This involves the use of trichloroacetic acid to produce the precipitate, digestion with perchloric acid and development with ammonium molybdate and aminonaphtholsulfonic acid reagent. Tests were read after 20 minutes had elapsed in a Bausch and Lomb spectrophotometer at 660 mμ wavelength. Each test was run in duplicate and occasionally in triplicate; each batch of serums tested at a given time included a normal control serum.

Three groups of individuals provided blood for these studies. The first group consisted of pregnant women treated with Rh hapten. The second was made up of nontreated pregnant women. The third consisted of nontreated, nonpregnant persons. For the first and second categories, bloods were sent in by air mail from various parts of the country and examination made within 24 hours after arrival. For the third group, bloods were drawn in the laboratory.

Altogether 44 tests for serum phospholipid were made on bloods drawn at biweekly intervals from five different pregnant women who were being treated. Determinations were obtained on bloods from eleven nontreated,

* Lewis, A. F. Unpublished observations.

pregnant women in the second and third trimesters of pregnancy, which is when the levels have been found to be highest, (Peters *et al.* 12). Fourteen serum phospholipid levels were determined on normal persons, of which eight were male and six were female. Since normal levels are so well established, it seemed unnecessary to investigate a larger number for this third group.

## *Results*

Fiske and SubbaRow (16) give normal lipid phosphorus values of between 8 to 10 mg. per 100 ml. of serum; Zilversmit and Davis provide a wider normal range of 6.1 to 14.5 mg. per 100 ml. serum. Table 1 shows the results obtained with serums

TABLE 1

*Mg. of lipid phosphorus/100 ml. serum*

| Woman (treated pregnant) | Fiske and SubbaRow | | | | | | |
|---|---|---|---|---|---|---|---|
| | Test 1 | Test 2 | Test 3 | Test 4 | Test 5 | Test 6 | Test 7 |
| A | 10.6 | 10.3 | 10.2 | 14.25 | 16.3 | 10.9 | 14.2 |
| B | 9.55 | 9.4 | 11.4 | ... | ... | ... | ... |
| C | 9.45 | 10.8 | N.C.C. | 12.6 | 11.2 | 12.5 | ... |
| D | 10.76 | 9.8 | 9.9 | ... | ... | ... | ... |
| E | N.C.C. | 12.2 | ... | ... | ... | ... | ... |
| | Zilversmit and Davis | | | | | | |
| | Test 8 | Test 9 | Test 10 | Test 11 | Test 12 | Test 13 | Test 14 |
| A | 16.3 | 18.65 | 17.8 | 18.6 | 18.6 | 18.3 | ... |
| C | 11.9 | 13.8 | 11.7 | 11.5 | 14.7 | 15.6 | 14.3 |
| D | 12.4 | 13.8 | 13.4 | 14.7 | 13.4 | 12.0 | ... |
| E | 11.6 | 11.9 | 16.1 | 8.9 | 12.3 | 11.7 | ... |

N.C.C. = No color change

Level of lipid phosphorus in biweekly serum samples of pregnant treated women as determined by methods of Fiske and SubbaRow and Zilversmit and Davis.

from pregnant, treated women. The mean obtained with the method of Fiske and SubbaRow is 11.3 mg. per 100 ml. serum, which is above their top normal limit. With the Zilversmit and Davis method the mean is 14.1 mg. per 100 ml. serum. This is about at top normal. Table 2 shows serum phospholipid levels with non-treated, pregnant women. The mean for this group, all tested by the method of Zilversmit and Davis, is 14.9 mg. per 100 ml. serum, just above top normal. Table 3 contains the results of tests on normal bloods, for which both methods were used. The mean for those tested by the method of Fiske and SubbaRow is

10.2 mg. per 100 ml. serum, barely above top normal. The mean for the serums tested by the method of Zilversmit and Davis is 9.0 mg. per 100 ml. serum, which is, of course, in the middle of normal range. It is obvious that, with both methods used, the pregnant women, whether treated or not, had higher phospholipid levels than did the normal individuals.

A fairly progressive rise in the lipid phosphorus values of the biweekly serum samples collected from pregnant, hapten treated

TABLE 2

| Woman (nontreated pregnant) | Zilversmit and Davis Mg. lipid P/100 ml. serum |
|---|---|
| F | 14.0 |
| G | 15.0 |
| H | 18.3 |
| I | 12.9 |
| J | 10.1 |
| K | 11.1 |
| L | 14.8 |
| M | 13.2 |
| N | 11.5 |
| O | 11.2 |
| P | 12.1 |

Level of lipid phosphorus in the serum of nontreated pregnant women in the second and third trimesters of pregnancy as determined by the method of Zilversmit and Davis.

TABLE 3

*Mg. of lipid phosphorus/100 ml. serum*

| Individual | Test 1 | Test 2 | Test 3 |
|---|---|---|---|
| | | Fiske and SubbaRow | |
| Q (female) | 9.17 | 9.07 | 8.5 |
| R (male) | 10.5 | ... | ... |
| S (male) | 9.45 | ... | ... |
| T (male) | 10.8 | ... | ... |
| U (female) | 12.3 | ... | ... |
| V (male) | 11.7 | ... | ... |
| | | Zilversmit and Davis | |
| Q (female) | 9.2 | 8.6 | |
| S (male) | 8.8 | 9.5 | |
| T (male) | 9.9 | ... | |
| W (female) | 8.2 | ... | |

Level of lipid phosphorus in the serum of normal individuals as determined by the methods of Fiske and SubbaRow and Zilversmit and Davis.

women can be observed. However, according to Peters and associates (12) this is to be expected from the twelfth week of pregnancy until term. In comparing these values against those obtained for pregnant, non-treated women, only three samples from one woman receiving the hapten exceeded the highest control value. The three samples, however, contained only 0.3 to 0.35 mg. of lipid phosphorus more than that found for the highest value from a non-treated woman. Deviations among duplicate or triplicate tests in any of the three groups never exceeded 0.2 mg.

## *Discussion*

There is no evidence that the ingestion of the Rh hapten in any way affects the serum phospholipid levels. A statistical comparison of the three groups revealed the following information. When the values for the treated, pregnant women were compared to values for the non-treated, pregnant women, it was found that $T = .21$ which is not significant. On the other hand, comparison of the treated pregnant women with normal individuals gives a $T = 4.26$. In addition, a comparison of non-treated pregnant women with normal individuals yields a $T = 4.80$. These findings, at a 1 per cent level of confidence, are highly significant. Therefore, no significant difference has been demonstrated in the serum phospholipid levels of pregnant women to whom hapten has been administered and of those receiving no hapten. The increase in these values in the treated, pregnant women apparently is due to pregnancy alone.

## *Acknowledgment*

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# NEUTRALIZING ANTIBODIES IN HUMAN SERA TO *HERPESVIRUS SIMIAE* (B VIRUS)[a]

GERALD L. VAN HOOSIER, JR. AND JOSEPH L. MELNICK[b]

*Herpesvirus simiae* (B virus) occurs in monkeys as a latent infection much as herpes simplex virus does in man (*Herpesvirus hominis*) (11). The two viruses are antigenically related. However, while B virus antiserum neutralizes herpes simplex and B viruses equally well (1), the degree of cross reactivity of herpes antiserum with B virus is not clear. Some workers (10, 9, 8) have reported that human sera are essentially unable to neutralize B virus, while others (7) found that a significant number were positive. When neutralization tests were carried out in infant mice (7), about 50% of sera neutralizing herpes virus were also able to neutralize B virus. Sera without herpes antibodies failed to neutralize B virus. Since a number of fatal B virus infections have occurred in recent years, the availability of a known virus inhibitory agent in the form of gamma globulin would be of more than academic interest. As most of the recent work on neutralization of B virus has been carried out in tissue culture, we have reinvestigated B virus neutralization using a plaque assay system, already described for B virus by Youngner (12).

## *Materials and Methods*

*Virus strains:* The first strain of B virus to be isolated directly from a monkey, by Melnick and Banker (7), was used. It was passed 2 times in rabbit kidney tissue cultures, and stock virus was stored at −70° C. in 50% skim milk for use in the tests. A strain of *H. hominis*, was kindly supplied by Kaplan (4); the virus was passed and stored in the same manner as the B virus.

*Tissue cultures:* Monolayers of monkey kidney tissue cultures (MKTC) and rabbit kidney tissue cultures (RKTC) were prepared in 2 oz. prescription bottles by methods described elsewhere (5). Growth media consisted of Medium 199 plus calf serum, 2% for MKTC and 10% for RKTC. The cultures

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were incubated for 6 to 8 days prior to inoculation. The agar-overlay medium was essentially the same as described for MKTC (5).

*Sera:* Blood samples were obtained from a group of men 25–47 years old, who had been in contact with rhesus and cynomolgus monkeys for a period of 1–5 years. The sera were stored at —20° C. until tested.

*Neutralization tests:* Diluent for virus and serum was Hanks' Balanced Salt Solution. Equal volumes of a virus dilution calculated to contain approximately 50–100 plaque forming units (PFU) under the test conditions were mixed with two-fold dilutions of sera and incubated at 37° C. for 1 hour. Aliquots of 0.2 ml of the virus-serum mixture were inoculated onto cultures of MKTC for B virus assays, and RKTC for herpes virus assays; cultures were incubated for 1 hour at 37° C. prior to addition of the agar overlay. Incubation was continued at 37° C. until the plaques were counted at 5–7 days.

## *Results*

A total of 13 human sera were tested for antibodies; a serum was considered positive if 80% or more of the plaques were neutralized. All sera were screened for antibodies to *H. hominis* at a dilution of 1:8 and all were positive. The sera were then tested for antibodies to B virus. Five sera showed no neutralization at a dilution of 1:4, three were positive at 1:4, three were positive at 1:8; and two were positive at 1:16. Fig. 1 shows the plaque bottles with one serum which had a titer of 1:8. It should be noted that there was one plaque at the 1:4 dilution of serum, and there was still some plaque reduction, as compared with the control bottle, with the 1:16 dilution of serum.

## *Discussion and Conclusions*

Previous unreported studies in this laboratory failed to demonstrate antibodies to B virus in these human sera when the conventional tube neutralization test was used. Application of the plaque technique has added considerable refinement to the assay of virus and antibody. In the present work with the plaque technique, even when a 1:8 dilution of serum was found to neutralize 80% of the PFU, one plaque could still be found at a 1:4 dilution of serum (Fig. 1). This might explain the negative results in the tube neutralization test, since one infectious particle could result in degeneration of all the cells in tube culture under fluid medium. With certain ECHO and Coxsackie strains, similar difficulties have been observed in the tube neutralization test, and these also were resolved by turning to the plaque neutralization test (6, 3).

FIG. 1. Neutralization of B virus by human serum. Top row: $10^{-2}$ dilution of B virus plus indicated dilution of serum. Serum titer equals 1:8. Bottom row: Control bottles. MKTC 6 days after seeding with $10^{-2}$ and $10^{-3}$ dilutions of virus.

The presence of low levels of neutralizing antibodies in certain human sera to B virus is probably the result of previous antigenic experience with *H. hominis*. Another possible explanation is that some of these individuals had experienced inapparent infection with B virus. However, the present results of 8 *H. simiae*-positive sera out of 13 *H. hominis*-positive sera tested are not significantly different from the 11 of 20 previously reported in normal persons (7).

A human vaccine against B virus is highly desirable and preliminary results in animals have been published (2). Until such a vaccine is proven safe and effective for humans, monkey handlers might be spared some degree of risk if they possessed antibodies which neutralized *H. simiae* as well as *H. hominis*. Human gamma globulin in large doses would also seem to be advisable for all persons receiving monkey bites (7). However, one person died of B virus infection in spite of the presence of herpes antibodies (8).

*Summary*

The plaque reduction technique on monkey kidney tissue cultures was adapted for measuring antibodies against B virus. It was found that sera of certain individuals are able to neutralize *Herpesvirus simiae* (B virus) as well as *Herpesvirus hominis* (Herpes simplex virus). Antisera of other individuals which also neutralize *Herpesvirus hominis* were found to be ineffective against B virus. This may indicate that strains of herpes virus of man differ somewhat in their antigenic composition, or it may be a reflection of differences in herpes antibody titer.

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# PLASMA PROTEINS OF SUSCEPTIBLE AND RESISTANT HOSTS OF THE FILARIAL WORM LITOMOSOIDES CARINII[a]

E. Ahmed Zein-Eldin and J. Allen Scott

The filarial worm *Litomosoides carinii* is naturally parasitic in the cotton rats *Sigmodon hispidus hispidus* and *Sigmodon hispidus texianus*. The adult male and female worms are located in the pleural cavity of the host, where the ovoviviparous females deposit their larvae, the microfilariae. These larvae enter the circulating blood and are taken up by a blood feeder, the tropical rat mite *Ornithonyssus bacoti*. In the mite the larvae penetrate into the hemocoel and undergo development through three morphological stages in about 18–20 days at a controlled temperature. After they have reached the third stage, the larvae may re-enter the cotton rat at a time of subsequent feeding of the mite. In the definitive host the larvae migrate to the pleural cavity, resume development, pass through three more morphological stages culminating in the adult stage. Microfilariae appear in the peripheral blood about 51 days after the infective larvae enter the rat. Initial infections of cotton rats induce an acquired immunity which results in a retarded growth rate of the worms of a superimposed infection (Macdonald and Scott 1953).

The mongolian gerbil *Meriones unguiculatus* has experimentally been found susceptible to infection with *L. carinii*. The worms attain maturity in a shorter period, but with a duration of infection shorter than in the cotton rat.

Adult white rats *Rattus norvegicus* are almost completely resistant to infection, in that few worms reach the pleural cavity and virtually all die before reaching maturity. Immature white rats, however, are susceptible (Olson 1959).

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Florida rice rats *Oryzomys palustris natator* are completely resistant, no worms having developed in a large number of animals tested in this laboratory.

Little is known about the factors involved in this type of natural immunity exhibited by the resistant hosts. In general it is not known whether the parasites find inhibiting factors in the so-called naturally immune hosts and a lack of such inhibition in normal hosts, or whether the normal host merely provides favorable nutritional material not present in other species. The study of the blood plasma proteins in the susceptible and resistant hosts of *Litomosoides carinii* has been undertaken in order to determine the presence or absence of certain fractions which might be linked to the unknown mechanism of natural immunity.

## *Methods*

In a preliminary investigation of the blood serum, it was found that the Texas and Eastern cotton rats have identical electrophoretic patterns. This eliminated the necessity for using the two subspecies and the Eastern subspecies was chosen to represent the cotton rats. A total of 27 Eastern cotton rats, 26 gerbils, 23 Florida rice rats, 30 mature white rats (over three months of age) and 6 immature white rats (20 days old) were examined. The use of serum invariably necessitated the sacrifice of the donor owing to the large blood sample required for the preparation of serum. Consequently, blood plasma was substituted.

Plasma samples were obtained from less than 0.1 ml. of blood per animal drawn from the tip of the tail into heparinized capillary tubes. The tubes were sealed at one end with modeling clay and centrifuged in a hematocrit centrifuge for five minutes. Using a pair of blunt scissors, the tubes were each cut into two lengths. The lower portions containing the blood cells were discarded. The plasma in the upper portions was recovered into micropipettes, from each of which a sample of 8 microliters was applied to each strip of filter paper in the electrophoresis apparatus. The plasma protein fractions were separated by the hanging strip method of paper electrophoresis in a Durrum cell (Block, Durrum, and Zweig, 1958). In almost every run 2 strips out of 8 in the Durrum cell were used for one species of host, to minimize the experimental error. Duration of each run was 16 hours at an initial direct current of 2 milliamperes and a final current of 5–7 milliamperes. Bromophenol blue dye at the strength of 0.1 gm per 100 ml. of purified methanol was used to stain the strips. The dry strips were scanned with the Spinco analytrol scanner and integrator. A mean percentage value was then established for each protein fraction in each species of host. These means were transformed into radians and compared to determine the significance of differences among these hosts. The analysis of variance test and the multiple range test (Kramer 1956) were used at a $P<.01$. The number of blood samples drawn from each animal ranged from 1 to 3, and were taken at different times.

A total number of runs for each species of animals was 72, 40, 50, 53 and 6, for cotton rats, gerbils, rice rats, mature white rats and immature white rats respectively.

## *Results*

Based on the number of protein fractions found in the blood plasma of the hosts, a consistent general difference is apparent between the two groups of hosts. The resistant hosts have seven fractions whereas the susceptible ones have only five fractions (cotton rats and immature white rats) or six fractions (gerbils). The seven fractions found in the plasma of the resistant hosts (rice rats and mature white rats) are fibrinogen (Ø), albumin, and the globulins which have been assigned the names $\gamma$, $\beta_2$, $\beta_1$, $\alpha_2$, and $\alpha_1$ (Figs. 3, 4, 5). Fibrinogen always appeared as an independent fraction except in one group of rice rats (group A) where for an unknown reason it overlapped with $\beta_2$ globulin in

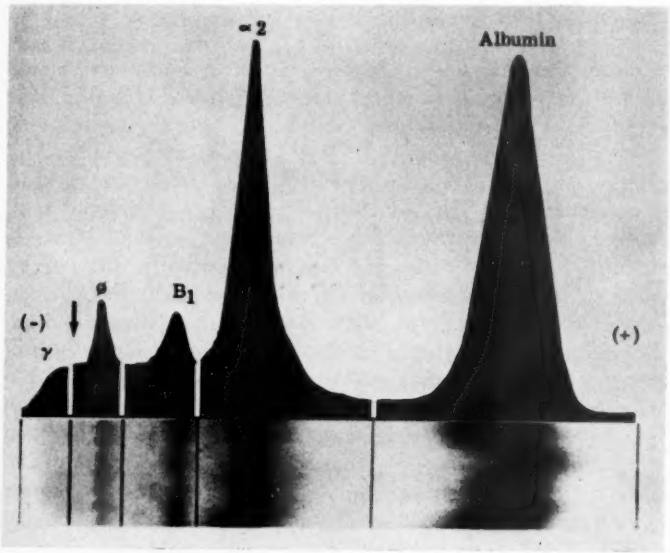


FIG. 1. Electrophoretic pattern of the cotton rat plasma.

The arrow in each figure points at the place where the plasma sample was applied to the paper strip.
(—) indicates the cathode side.
(+) indicates the anode side.

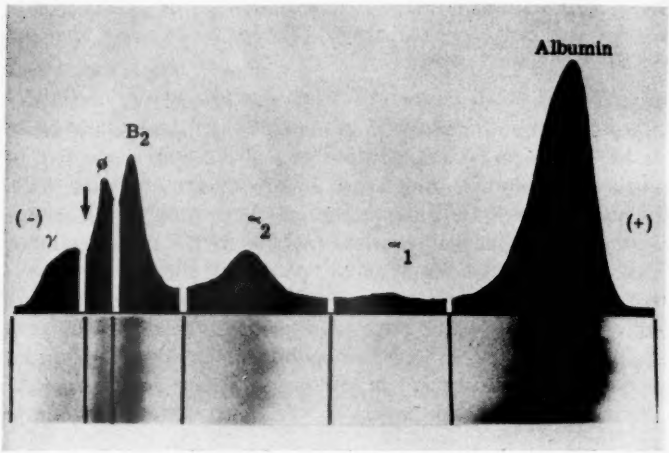


FIG. 2. Electrophoretic pattern of the gerbil plasma.

a common fraction termed "Ø + $\beta_2$" (Fig. 3). The cotton rat, the natural host, showed a complete lack of $\beta_2$ and $\alpha_1$, globulins, thus exhibiting only five fractions (Fig. 1). The gerbil, a susceptible host, showed a complete lack of $\beta_1$ globulin and exhibited six fractions, one of which ($\alpha_1$ globulin) occurred in a very small amount (Fig. 2). The immature white rat, a susceptible host, showed only five fractions, one of which represents $\beta_1$, $\alpha_2$, and $\alpha_1$ globulins combined (Fig. 6). The area normally occupied by these three individual fractions in the mature white rat (Fig. 5) appeared in the immature white rat as a homogeneous and undifferentiated zone. Hence identity or measurement of each of these three fractions was made impossible and they were considered as a single complex fraction.

Comparing the percentage values quantitatively, the fraction with cathodal migration designated as $\gamma$ globulin occurs in statistically significantly lower amounts in the cotton rats, a number of rice rats (group B in Table 1) and immature white rats than in the gerbils, mature white rats and Group A of rice rats (Table 1). The significantly higher $\gamma$ globulin content in the mature white rats as compared to immature ones is presumably due to the former having been exposed in the course of their longer life to many more antigens than the latter. Based on the

γ globulin fraction, therefore, no specific delineation of susceptible and resistant hosts could be attempted. Table 1 shows that fibrinogen occurs in the high percentage of 7.74 in the gerbil, an amount statistically significantly higher than all other hosts. One of the resistant hosts (the mature white rat) shows the second

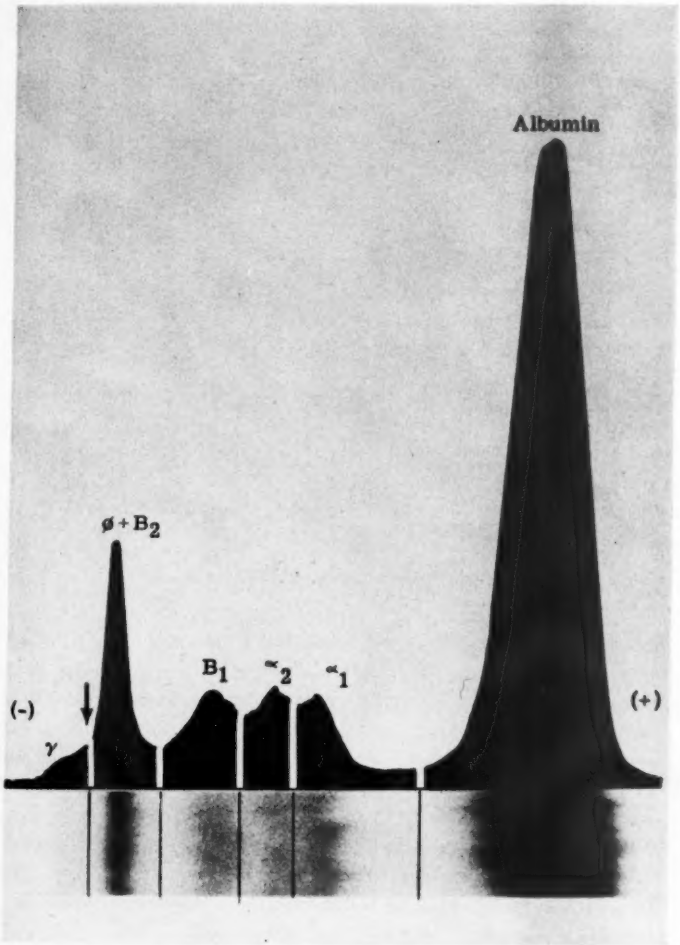


FIG. 3. Electrophoretic pattern of group A rice rats plasma.

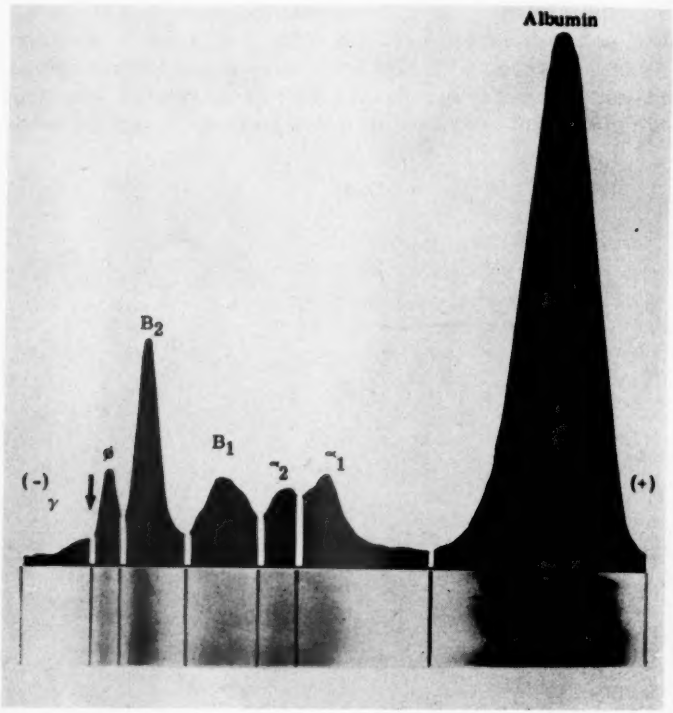


FIG. 4. Electrophoretic pattern of group B rice rats plasma.

highest value of 6.47% for fibrinogen, which is not statistically significantly higher than that of 5.76% found in the natural host, the cotton rat. It is of interest to point out that in view of the values obtained for fibrogen, no distinction is apparent between susceptible and resistant hosts. This finding is in agreement with the fact that fibrinogen makes no contribution to the known mechanisms of immunity.

Based on the values of $\beta_2$ and $\beta_1$ globulins in Table 1 no distinction was found between susceptible and resistant hosts. The natural host lacks the $\beta_2$ globulin fraction while the other susceptible hosts, the gerbil and the immature white rat show this fraction at percentages of 15.99 and 23.61 respectively. As to the $\beta_1$

globulin in the susceptible hosts, the cotton rat shows a content of 9.77%, while the gerbil contains none and the immature white rat does not exhibit this fraction in an identifiable manner. The mature white rat, a resistant host, shows $\beta_2$ and $\beta_1$ globulins at percentage values of 18.44 and 4.38 respectively. The other resistant host, the rice rat shows these fractions at values of 10.68% and 9.43% respectively in the group of animals where $\beta_2$ globulin and fibrinogen were separable (group B rice rats. Table 1). Group A of rice rats shows a $\beta_1$ globulin content of 10.81%, while the value of $\beta_2$ globulin could not be ascertained due to overlapping by the fibrinogen fraction.

The only consistent and statistically significant difference to which resistance to this infection may possibly be attributed

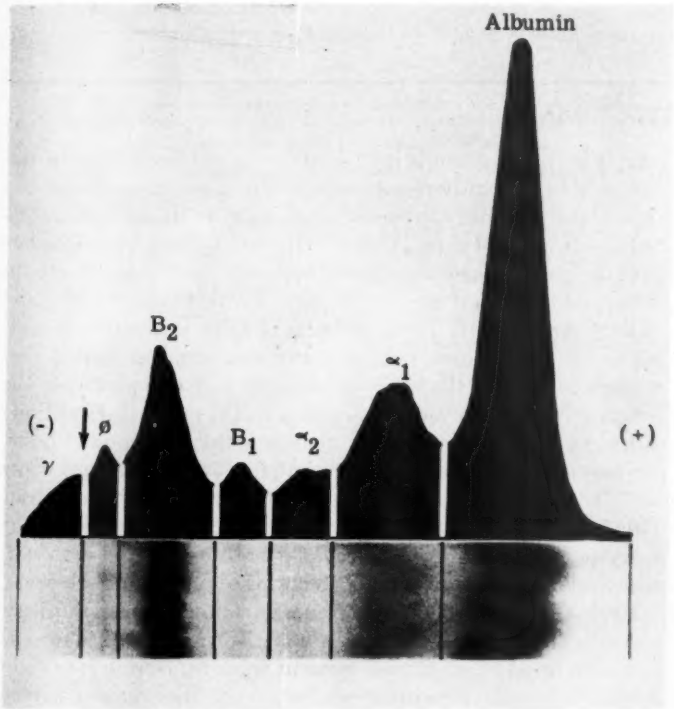


FIG. 5. Electrophoretic pattern of the mature white rat plasma.

TABLE 1

*Mean percentage values of plasma protein fractions of the hosts*

| Host | N | Percent protein fractions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $\gamma$ glob. | Fibrinogen $\phi$ | $\phi+\beta_2$ | $\beta_2$ glob. | $\beta_1$ glob. | $\alpha_2$ glob. | $\alpha_1$ glob. | Albumin |
| Cotton rats (27) | 72 | 2.59 | 5.76 | .... | .... | 9.77 | 30.16 | .... | 50.89 |
| Gerbils (26) | 40 | 3.86 | 7.74 | .... | 15.99 | .... | 14.29 | 4.97 | 53.16 |
| Rice rats | | | | | | | | | |
| Group A-13 | 33 | 3.17 | ... | 13.79 | .... | 10.81 | 5.54 | 9.73 | 56.98 |
| Group B-10 | 17 | 2.39 | 4.75 | .... | 10.68 | 9.43 | 4.65 | 10.23 | 57.85 |
| (23) | 50 | 2.91 | | | | 10.36 | 5.24 | 9.92 | 57.27 |
| White rats (over 3 months old) (30) | 53 | 3.41 | 6.47 | .... | 18.44 | 4.38 | 4.63 | 15.05 | 47.62 |
| White rats (20 days old) (6) | 6 | 2.66 | 4.12 | .... | 23.61 | Undifferentiated | | | 56.49 |
| | | | | | | | 13.14 | | |

Figures in parentheses under Host refer to total number of animals tested in each case.
N refers to total number of runs in each case.

resides in the $\alpha$ globulins. The two resistant hosts show a statistically significantly lower content of $\alpha_2$ globulin and similarly significantly higher content of $\alpha_1$ globulin than the two susceptible hosts. Table 1 shows that the rice rats and mature white rats have $\alpha_2$ globulin percentages of 5.24 and 4.63 respectively, as compared to the cotton rats and gerbils which have 30.16% and 14.29% respectively. The $\alpha_1$ globulin fraction is completely missing in the natural host, the cotton rat, and occurs at the low percentage of 4.97 in the other susceptible host, the gerbil. In the resistant hosts, however, this fraction occurs at a statistically significantly higher percentage than in the susceptible hosts; namely 9.92% in the rice rat and 15.05% in the mature white rat. The case of the susceptible immature white rat is less clear. This is due to the fact that a combined homogeneous zone in the analysis of immature white rat plasma occupies the area normally occupied by $\beta_1$, $\alpha_2$ and $\alpha_1$ globulins of the mature white rat.

Table 2 summarizes the differences found among the hosts and whether these differences are statistically significant. The hosts are listed opposite each protein fraction, from left to right in the order of increasing percentage of the given fraction. Wherever a common line is drawn under two or more hosts this

indicates that these hosts are not significantly different from one another, and may be considered as having been drawn from the same population. The hosts which do not share a common underline, on the other hand, may be considered as belonging to different populations. The significance of differences shown by underlining was determined at a P value $<.01$ using the analysis of variance and the multiple range test cited under Methods.

## *Discussion*

The term "Resistance" is used here to refer only to the actual failure to infect the host, and not to the host resistance to the effects of infection. The hosts which are called "resistant" are those apparently possessing certain inborn qualities or potentials which cause a fundamental incompatibility between the host and the parasite. These qualities are independent of past infections. This type of natural resistance may be due to one or more of

TABLE 2

*Differences among hosts and their statistical significance based on the percentage values of the plasma protein fractions*

| Protein fraction | Lowest % → Hosts → Highest % | | | | |
|---|---|---|---|---|---|
| $\gamma$ globulin | CR | WR | RR | WR+ | G |
| Fibrinogen | WR | RR | CR | WR+ | G |
| $\beta_2$ globulin | CR(Nil) | RR | G | WR+ | WR |
| (X)$\beta_1$ globulin | G(Nil) | WR+ | CR | RR | |
| (X)$\alpha_2$ globulin | WR+ | RR | G | CR | |
| (X)$\alpha_1$ globulin | CR(Nil) | G | RR | WR+ | |
| Albumin | WR | CR | G | WR | RR |

CR = cotton rat.
RR = rice rat.
G = gerbil.
WR = immature white rat.
WR+ = mature white rat.

(X) This fraction indistinguishable in immature white rats.

many causes ranging from a purely physical barrier to a highly selective nutritional requirement on the part of the parasite. The case of a physiological strain of *Litomosoides carinii* which occurs in the Georgia rice rat *Oryzomys palustris palustris*, but does not infect the Florida subspecies of rice rat *O. palustris natator*, possibly involves such a nutritional factor.

The association of nutrition with resistance to helminths has long been known (Ackert and Nolf, 1931), (Ackert and Beach, 1933), (Hager, 1941) and (Chandler, 1943).

In the present work, however, the factors affecting the susceptibility or resistance of a host are presumably attributable to the genetic constitution of infection-free hosts and not to the diet of the host. It has been found that, unlike the resistant hosts, the susceptible hosts are apparently capable of producing and maintaining a higher level of $\alpha_2$ globulin fraction, with the natural host, the cotton rat, still having twice as much of this fraction as the gerbil, the substitute host. It is hypothesized that this fraction may contain a large enough concentration of a selective nutritional source required by the migrating larvae as well as the adults of *L. carinii*.

Natural immunity has also been proved to have mechanisms at least partly similar to those operating in acquired immunity, i.e., antibodies. Terry (1957) found that a factor associated with $\beta$- and faster moving $\gamma$-globulins exists in the serum of the cotton rat, and is "produced by the antibody-forming apparatus without there being any external stimulus." This factor agglutinates and lyses *Trypanosoma vivax*.

In view of Terry's finding, it is suggested that a similar factor may be influencing the course of infection with *L. carinii* in the resistant hosts. Such a factor may reside in the $\alpha_1$ globulin fraction. This fraction is found in much larger amounts in the mature white rats and the rice rats than in the gerbil, and is completely absent in the natural host (Table 1). It is also possible that most of the undifferentiated zone in Fig. 6 of the immature white rat contains an increased amount of $\alpha_2$ globulin at the expense of $\alpha_1$ globulin. It may be true that the reverse occurs in the mature white rats, and that the $\alpha_1$ globulin is elaborated at the expense of $\alpha_2$ globulin as the rat advances in age. If indeed an anti-*Litomosoides* factor should later be found in the $\alpha_1$ globulin of the resistant hosts, such a finding would be compatible with the

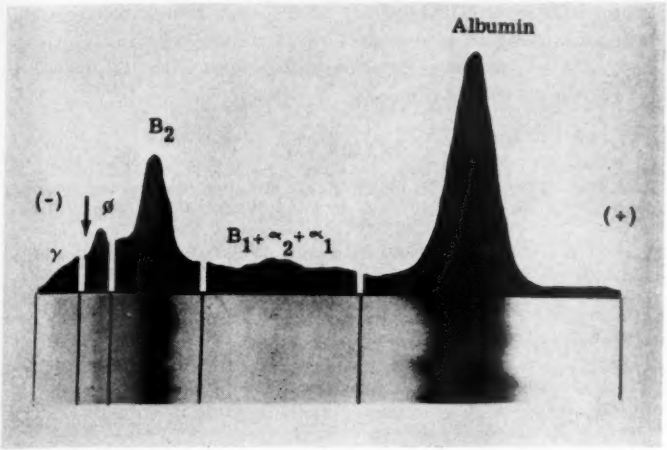


FIG. 6. Electrophoretic pattern of the immature white rat plasma.

postulation of antibody production without external stimulation.

Although these differences between hosts may be merely coincidental, they do seem to be somewhat correlated with the observed specificity of infection and may very well be playing an important role.

## *Summary*

Certain inherent differences have been demonstrated in the blood of four species of laboratory rodents whose susceptibilities to the infection with the filarial worm *Litomosoides carinii* are known. These animals include the cotton rat, *Sigmodon hispidus hispidus*, the natural host of *L. carinii*, the mongolian gerbil, *Mariones unguiculatus*, a susceptible host, the Florida rice rat, *Oryzomys palustris natator*, a highly resistant host, and the white rat, *Rattus norvegicus*, which is susceptible as an immature animal but becomes resistant as it grows older.

The protein fractions of the blood plasma of all these hosts were separated electrophoretically and identified. The percentage in which each fraction occurs was calculated and a mean value established for each fraction in each host. Statistically significant differences between the susceptible and resistant hosts have been found in the natural plasma of these infection-free hosts. Other

equally significant differences have been found between the plasma composition of mature and immature white rats. An attempt is made to correlate these differences with the degree of host resistance to this infection.

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# NOTES

## GROWTH OF A STRAIN OF ENTAMOEBA HISTOLYTICA AT ROOM TEMPERATURE[a]

DONALD A. DREYER[b]

The Laredo strain of *Entamoeba histolytica* was originally isolated by Dr. Frank H. Connell in 1956 from the stool of a patient from Laredo, Texas who complained of repeated episodes of diarrhea, weight loss, easy fatigability, and epigastric pain. *Entamoeba histolytica* was found as trophozoites by direct microscopic examination and was cultured in Nelson's medium. A few typical *E. histolytica* cysts appeared in the original and first subculture after four days growth. No other remarkable characteristics were observed at that time. In 1958, however, Dr. Connell noted that this strain could be cultured at room temperature.

The effect of temperature on survival and growth of *E. histolytica* has been studied extensively. Thermal death points (1, 14, 23) as well as cyst survival at low temperatures (4, 5, 11, 20, 21) have been reported. The effect of temperature on the trophozoites of *E. histolytica* has been studied by many workers (2, 3, 6, 7, 8, 9, 10, 12, 13, 16, 17, 19, 20) but none have been able to obtain a continuous culture at room temperature.

Successful cloning of the Laredo culture was accomplished by the following procedure: a 48 hour test tube culture of amoeba was serially diluted with media and dispensed dropwise on a sterile miscroscope slide until a single drop was found to contain only one trophozoite. The trophozoite was picked up with a micropipette using a Chambers Manipulator. The pipette was examined under the microscope to confirm the presence of only one amoeba. The amoeba was then placed in a test tube con-

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taining 10 ml of media. This procedure is similar to the cloning procedure recently reported by Rees, Key, and Shaeffer (18); however, they were unable to establish a clone without the use of a microtube. The clone culture was negative for amoeba on the 2nd and 4th days of incubation at room temperature but was positive on the 9th day with luxuriant growth. This strain, designated Laredo-2, was used throughout the present experiments. The bacterial flora that accompanies the Laredo-2 strain is that part of the original crude flora that was picked up with the micropipette at the time of cloning.

The culture medium used throughout this study was an all liquid medium similar to that of Moan (15) and consisted of:

| | |
|---|---|
| Liver extract concentrate powder 1:20 (NBC) | 2.0 gm |
| Disodium phosphate $Na_2HPO_4$ | 2.1 gm |
| Potassium phosphate (di-hydro) $KH_2PO_4$ | 1.6 gm |
| Sodium chloride NaCl | 3.0 gm |
| Bacto proteose peptone | 1.7 gm |
| Distilled water | 1000 ml |

The ingredients were combined and heated to just below the boiling point then allowed to cool to room temperature. Ten ml of Bacto cholesterol 1% in alcohol (100 mgm of cholesterol) were added with stirring and a slight precipitate formed immediately. The medium was then dispensed into 16 × 150 mm screw-cap tubes in 10 ml amounts and sterilized in the autoclave. Just before use, a small amount (approximately 10 mgm/tube) of sterile rice powder was added with a spatula. The medium was found to be stable for months in the refrigerator.

To transfer the amoeba, approximately one ml of sediment and fluid from the bottom of a culture tube was dispensed equally into two tubes of fresh media, then incubated on the laboratory shelf at room temperature, which was uncontrolled and ranged from approximately 10°C to 35°C. Transferring 2–3 times per week gave luxuriant growth, one transfer per week was adequate for good growth, and one transfer every 2–3 weeks was adequate to maintain the culture. Some cultures remained positive for over 6 weeks without transfer or addition of fresh media.

The Laredo strain of *Entamoeba histolytica* develops into a much richer culture than other strains seen by the author. At times, a drop of sediment from the bottom of a culture tube contains so many amoeba that they resemble an epithelial sheet of cells in tissue cultures (Fig. 1).

At present, the Laredo strain has been in culture over 4½ years and has been in culture at room temperature over 2½ years.

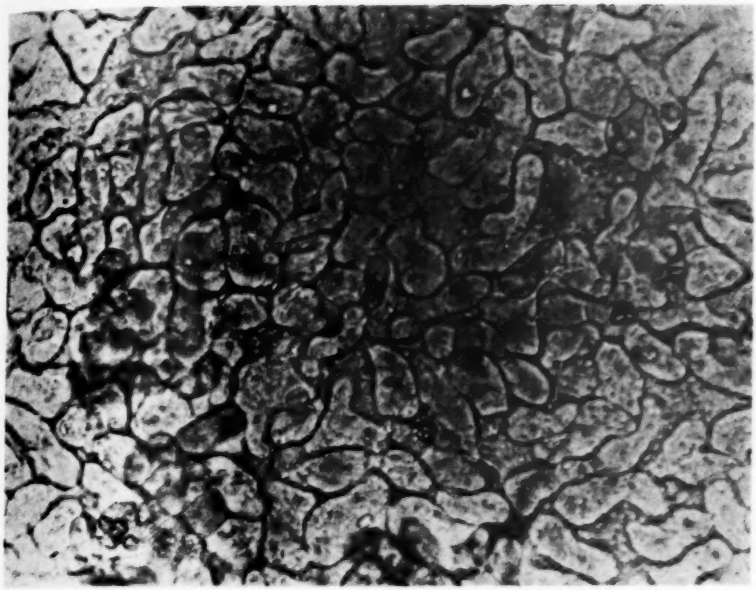

FIG. 1. *E. histolytica*, Laredo strain culture showing resemblance to an epithelial sheet, 430X.

The clone (Laredo-2) has been in culture at room temperature over 2 years without the necessity of a rigid schedule of transferring. The ease at which this strain can be cultured should make it a valuable aid in teaching and research.

*Acknowledgment*

The author is indebted to Dr. Frank H. Connell not only for a culture of the Laredo strain but for the information on its background.

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## BOOK REVIEWS

### Regulation of the Inorganic Ion Content of Cells. Ciba Foundation Symposium. Ed. by G. E. W. Wolstenholme and C. M. O'Connor. Little, Brown & Co., Boston, 1960. 100 pp.

This booklet consists of a compilation of six reports by E. J. Conway, H. H. Ussing, R. D. Keynes, W. Wilbrandt, A. Rothstein and P. C. Caldwell, plus the discussion following each of those papers. It is refreshing to see tedious but steady progress in the field of active transport presented lucidly by some of the major contributors in this field. What is more important, however, is this opportunity to observe the process of mental gymnastics that these investigators underwent during the discussion periods. The book is well indexed, has a fair bibliography, and is recommended to those people interested in an advanced treatise on active transport across membranes.

*S. N. Kolmen*

### Cellular Aspects of Immunity. Ciba Foundation Symposium. Ed. by G. E. W. Wolstenholme and C. M. O'Connor. Little, Brown & Co., Boston. 459 pp. with 117 illus. $10.50.

This book records the proceedings of a symposium, participated in by 32 highly qualified biologists, among whom are such familiar names as Burnet, Lederberg, Frageus, Medawar, Dixon, and Monod. Indeed, it is unfair to omit the complete list of important contributors to this volume.

This book presents considerable support for the concept that the immunologically competent cell, rather than antibody, has become the central theme of immunology, and that the immunologically competent mesenchimal cells may have any one of at least three morphological appearances: lymphocyte, histiocyte, and plasma cell. Some inquiries on "immunological memory" are discussed in seeking explanations of how immunological information is conveyed to an immunologically competent cell and how it is transmitted over long periods of time. This occurs presumably by a process of cellular descent.

The field is thoroughly presented in 20 papers and expanded through well edited pertinent discussions. An author and subject index add to the usefulness of this volume.

*Morris Pollard*

### The Social Epidemiology of Mental Disorders, by E. Gartly Jaco. Russell Sage Foundation, New York, 1960, 228 pp. $3.50.

This work, as stated in the subtitle, is a Psychiatric Survey of Texas. It is a presentation, analysis, and interpretation of data pertaining to sociological aspects of the psychotic population of Texas during the study period of 1951–52. Importantly, the data obtained by the author is from private and public hospitals as well as from files of psychiatrists in private practice; and the data presented is concerned with incidence rates, or number of *new* cases of a

disease during the study period, rather than prevalence rates, which are based on the number of *active* cases of a disease during a time period.

Following an introductory chapter which includes a general discussion of social surveys of mental illness and a more specific description of the scope and design of this particular study, the book is divided into four parts. Part one concerns the demographic aspects of mental disorders and variabilities of sex, age, migration and subculture are discussed in relation to the findings of the survey. Significant divergences were obtained in the incidence rates of mental disorders in terms of age, sex and subcultural composition that these factors were controlled in analyzing the data of the remaining variables of the study. Migration, however, was not found to be an important factor in the incidence of psychosis. Part two of the book concerns the social ecology of mental disorders, and the factors analyzed here were the spatial distribution of psychotics and urban-rural differentials. Again, significant variability in the incidence rate of psychosis was found based upon these ecological factors. Part three discusses the socioeconomic aspect of the psychotic population and introduces the factors of marital status, occupation, and education and their effect upon the incidence rates of psychosis. Following these major parts there is a summary and discussion of the previously presented data and this in turn is followed by appendices and a comprehensive bibliography.

In general the material is presented in an organized, comprehensible manner, although with a large number of variables and diagnostic categories, the data presented may tend to be heavily concentrated in places. However, this small shortcoming is overcome well by the liberal use of charts and general summaries at the chapter ends.

The evident care used by the author in his collection of data and interpretation of it plus the size and scope of the survey itself make this an important contribution to our understanding of the sociological factors of mental illness.

*John B. King, M.D.*

# CALLING ATTENTION TO:

## DECEMBER, 1960

*Reviews* are becoming increasingly necessary for interested scientists & physicians as a way by which to keep well oriented with current advances in areas of knowledge with which they may be concerned. Maybe we need as many thinking & library workers as bench & clinic investigators. Some recent important reviews for holiday reading follow. M Cattell edits symposium on cardiovascular effects of nicotine & smoking (*Ann NY Acad Sci*, 90/1–344/Sept 27/60). H Florey analyzes coronary artery disease (*Brit Med J* 2/1329/Nov 5/60). O Fresen reviews the *retothelial* (sensible for reticulo-endothelia system (*Deutsch Med Wchnschr*, 85/2009/60). DL Fry & RE Hyatt go over pulmonary mechanics in regard to pressure, volume & flow in healthy & sick people (*Amer J Med*, 29/672/60). A Grollman edits helpful discussion on new diuretics & antihypertensive agents (*Ann NY Acad Sci*, 88/771–1070/Oct 11/60). PR Gross edits symposium on mechanisms of cell division (*Ann NY Acad Sci*, 90/345–613/Oct 7/60). GJ Hamwi & Co review acromegaly (*Amer J Med* 29/690/60). FH Hoffman & WA Steiger analyze medical interviews with patients (*Postgrad Med* 28/315/60). JT Litchfield enjoys reviewing sequential analysis, drug screening & occasional serendipity (*J Med Pharm Chem* 2/469/60). WR Meadows reviews post-partum heart disease (*Amer J Cardiol* 6/788/60). GE Murphy reviews the nature of rheumatic heart disease (*Med* 39/289/60). B Roueche has another keen medical commentary, this time on the placebo (*New Yorker* Oct 15/60, pp85 $ ). R Rudnick edits 18th Growth Symposium on *Developing Cell Systems & Their Control* (Ronald Press, New York 10, 1960, 126pp, 240 illus, $8). RJ Schain reviews neurohumors of cerebrospinal fluid (*Yale J Biol Med* 33/15/60). FO Schmitt well discusses relations of molecular biology to medicine (*Bull NY Acad Med* 36/725/60) S Schotz opens symposium on anesthesiology in cardiac survey (*Amer J Cardiol* 6/705–787/60). J Tepperman & JR Brobeck open symposium on energy balance in animals, which contains much on appetite (*Amer J Clin Nutri* 8/527–774/60). UK & USA joint report on rheumatic heart disease in children treated with ACTH, cortisone, & aspirin, appears simultaneously (*Brit Med J* 2/1033/Oct 8/60 & *Circula* 22/503/60). PP Van Arsdel & GN Beall describe metabolism & function of histamine (*Arch Int Med* 106/714/60). SA Waksman edits symposium on actinomycins in treating tumors (*Ann NY Acad Sci* 89/283–486/Oct 5/60). CG Zubrod reviews drugs useful in treating cancer (*Arch Int Med* 106/663/60).

*Radiation Reviews:* AA Buzzati-Traverso edits important symposium on *Immediate & Low Level Effects of Ionizing Radiations* (Taylor & Francis, London EC 4, 1960, 381pp, $8). JS Mitchell is vastly informative, wise & modest in his *Studies in Radiotherapy* (Harvard Univ Press, Cambridge, Mass, 1960, 281pp, $9).

*Preclinical:* MR Alioto gives glutamine-pyruvic transaminase content of various tissues (*Ital J Biochem*, 9/199/60). JE Gray & Co find acetyl salicylic

acid anhydride non-irrating to stomach (*Toxicol Appl Pharmacol*, 2/514/60). TN Nesmeianova & Co confirm WE Windle (*Physiol Rev* 36/427/56) that regeneration of nerves in spinal cord occurs if scarring is delayed by pyrogens (*Sechenov Physiol J USSR* 46/238/60). CM Rangan & RR Bhagwat report on hormone effects on human red cell sedimentation rates (*Indian J Med Res*, 48/549/60). E Sicuteri finds anti-epileptic action in a serotonin-antagonizing lysergic acid derivative (*Med Experi* 2/36/60). LN Shimkin finds stimulation of intestinal epithelial mitosis after pain stimulation of the tongue (*Doklady Acad Nauk USSR* 134/694/60).

*Pertinent Holiday Reading*: C Bibby biographizes *TH Huxley: Scientist, Humanist & Educator*, with comments by grandsons Julian & Aldous (Horizon Press, New York, 1960, 330 pp, $5) EL DuBrul gives structural evidence in the brain for a theory of the evolution of behavior (*Perspect Biol Med*, 4/40/60). DL Gilbert speculates on relation between organic & atmospheric evolution (*ibid* p 58). CG Hartman asks many questions on mechanisms of conception (*Ibid* p 77). Sir Julian Huxley describes the emergence of Darwinism (*Ibid* 3/21/60). O Loewi offers a delightful pharmacologic autobiographical sketch (*Ibid* 4/3/60). Why not subscribe to *Perspectives in Biology & Medicine* edited by DJ Ingle for Univ Chicago Press, & enjoy good reading? ML Johnson-Abercrombie exposes *The Anatomy of Judgment: An Investigation into the Processes of Perception & Reasoning* (Basic Books, New York, 1960, 156 pp, $4.5). W McClure is provocative & logical in *World Legal Order: Possible Contributions by the People of the United States* (University of North Carolina Press, Chapel Hill, 1960, 366 pp, $7.5). S&P Ottenberg edit *Cultures & Societies of Africa* (Random House, New York, 1960, 614 pp, $7.5). P Ritner shouts a warning in *The Death of Africa* (Macmillan, New York, 1960, 232 pp, $5). DW Sciama describes *The Unity of the Universe; Our Evolving View of the Cosmos* (Doubleday & Co, Garden City, NY, 1960, 228 pp, $4).

JANUARY, 1961

*Notable New Books*: D Abelson & RV Brooks edit *Paper Chromatography of Steroids* (Cambridge Univ Press, London, 1960, 103 pp, $6). R Brown deals with language as human behavior in *Words & Things* (Free Press, Glencoe, Ill, 1960, 398 pp, $6.75). M Florkin of Liege ponders *Unity & Diversity in Biochemistry* (Trans by T Wood for Pergamon Press, London, 1961, 400 pp, $10). M Kleiber analyzes *The Fire of Life: An Introduction to Animal Energetics* (Wiley, New York 15, 1961, 267 pp, $6). HD Levine advises on *Cardiac Emergencies & Related Disorders* (Appleton-Century, New York 1, 1960, 368 pp, $12). MM Nierman's *Patients & Ponies* is fun (Random House, New York, 1960, 173 pp, $3.5). R Spencer integrates informations on *The Intestinal Tract: Structure, Function & Pathology* (CC Thomas, Springfield, Ill, 1960, 424 pp, $13). WP True skillfully edits fascinating series of Smithsonian Reports, from the structure of the universe to HG Wells' discovery of the future, reprinting many classics from 1897 to date (Simon & Schuster, New York, 1960, 1208 pp, in 3 vols, $15). L Uhr & JC Miller edit integrated reports on theories, methods & results of behavioral research on effects of

psychoactive agents in *Drugs & Behavior* (Wiley, New York 16, 1960, 676 pp, $11). GEW Wolstenholme & CM O'Connor edit Ciba Foundation Study in honor of EJ Conway on *Regulation of the Ion Content of Cells* (Little Brown, Boston, 1960, 100 pp, $3).

*Clinical*: BD Burns & JD DeJong report on measurement of Parkinson's disease (Neurol 10/1096/60). GHA Clowes Jr reviews extracorporeal maintenance of circulation & respiration (*Physiol Rev*, 40/826/60). L Goldberg & Co report iron overloading phenomena (*Toxicol Appl Pharmacol* 2/683/60). On *leukemia*, note important series of reports by WM Court-Brown & Co (incidence after exposure to diagnostic radiation in utero); EE Pochin (following radio-iodine treatment); TLT Lewis (following diagnostic radiation); RHD Bean (phenyl-butazone) & S Shuster & Co on fetal hemoglobin (*Brit Med J* 2/1539; 1545; 1551; 1552; 1556, Nov 26/60). For international aspects of current cancer incidence & control, leukemia, & cancer education see (*Unio Internat contra Cancera Acta* 16/1509–1875/60). SX Radbill & GR Hamilton discuss fact & fancy in measles (*Bull Hist Med*, 34/430/60).

*Preclinical*: RK Crane reviews intestinal absorption of sugars (*Physiol Rev*, 40/789/60). JG Defares & Co analyze cerebral blood flow in regulation of respiration (Acta Physiol Pharmacol Neerland 9/327/60). J Holubar studies EEG changes in skin galvanic responses, & also photic driving (Physiol Bohemoslov 9/472, 477, 482/60). J Grayson & D Mendel note vascular & metabolic brain changes after insulin & glucose (J Physiol 154/26/60). P Kovacic proposes role for hydrogen peroxide in carcinogenesis (*Ohio J Sci*, 60/257/60). GP Lewis summarizes data on active polypeptides derived from plasma proteins (*Physiol Rev*, 40/647/60). AA Mazuroh finds lactate reduces vagal heart action & enhances adrenergic effects (*Sechenov Physiol J USSR* 46/378/60). WR McCabe critically appraises furaltadone (*New Eng J Med*, 263/927/Nov 10/60). MW Olsen summarized 9 years of parthenogenesis in turkeys (*Proc Soc Exp Biol Med*, 105/279/60). RE Olsen & JW Vester discuss nutrition-endocrine relationships in controlling fat transport in humans (*Physiol Rev*, 40/677/60). PG Reizenstein & Co find lymphatics not involved in intestinal absorption of iron or Vitamin $B_{12}$ (*Proc Soc Exp Biol Med*, 105/233/60). S Spector & Co show monoamine oxidase is factor in norepinephrine metabolism in brain (*J Pharmaco Exp Therap*, 130/256/60). J Strauss & GG Nahas find tris-hydroxymethyl-aminomethane (THAM) increases salicylate excretion (*Proc Soc Exp Biol Med*, 105/348/60). S Thesleff analyzes effects of motor innervation on the chemical sensitivity of skeletal muscle (*Physiol Rev*, 40/734/60). L D Wright & Co note bicarbonate inhibition of ATP levels necessary for cholesterol synthesis in liver (*Proc Soc Exp Biol Med*, 105/383/60).

*General*: VN Andreeva relates how Pavlov's work in Botkin's clinic prepared way for studies on gastric reflex effects & for pharmacology (*Sechenov Physiol J USSR* 46/421/60). A Berman gives keen account of Russian military medical formularies 1765–1840 (*Amer J Hosp Pharm*, 17/211/60). JB Conant reflects on history in the education of scientists, paying tribute to

George Sarton (*Amer Sci*, 48/528/60). LJ Rather writes well of Jerome Gaub (1705–1780) of Leiden who had idea of psychosomatic medicine (*Stanford Med Bull*, 18/111/60). H Shapley edits Star Island symposium on *Science Ponders Religion* (Appleton-Century, New York, 1960, 308 pp, no index, $5). CW Waddington contemplates *The Ethical Animal* (Allen & Unwin, London, 1960, 230 pp, 25 s). K Wallace tells of George Heye (1874–1957) who ran great Heye Foundation Museum of the American Indian, Audubon Terrace, NY, *New Yorker*, Nov 19/60, p 104 ff).

C. D. Leake